Characterization of *Stachybotrys elegans*' genes regulated during its interaction with *Rhizoctonia solani*

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ABSTRACT

Stachybotrys elegans is a mycoparasite of the soilborne plant pathogen fungus *Rhizoctonia solani*. The mycoparasitic activity of *S. elegans* is correlated with the production of cell wall-degrading enzymes such as chitinases. This study details the cloning and characterization of the cDNA, *sechi44*, that encodes an extracellular endochitinase. The expression regulation of *sechi44* was altered when *S. elegans* was in interaction with its host, *R. solani*, and also when the mycoparasite was grown on minimal media amended with different carbon and nitrogen sources. Direct contact with *R. solani* significantly upregulated *sechi44* expression which followed a cyclical pattern suggesting that this gene has a role not only in mycoparasitism, but also in linear growth of the mycoparasite. The addition of high concentrations of glucose and ammonium triggered a decrease of *sechi44* expression suggesting that *sechi44* is subject to glucose and ammonium repression. In a separate study, several genes (1016 clones) whose transcription was substantially up-regulated during the mycoparasitic interaction were identified using SSH and microarray analysis. Twenty-five percent (261 clones) of these were sequenced and assigned to putative functions. Among them, 15 expressed sequence tags (ESTs) were identified in *R. solani* whose functions were related to defense while the majority of ESTs were identified in *S. elegans* and assigned functions related to toxin metabolism, pathogenic process, stress response, multidrug resistance, apoptosis, transport, ATP synthesis, replication, transcription and DNA repair, translation, transduction, protein degradation, and ribosomal protein. The overexpression of 13 selected genes of *S. elegans* was validated and confirmed using quantitative reverse transcription polymerase chain reaction (QRT-PCR). The temporal gene expression of nine genes was monitored when the mycoparasite was grown on *R. solani* (host) and *Sclerotinia sclerotiorum* (non-host) mycelia and sclerotia. Some genes such as *seglu*, *selec*, and *se151* were completely inhibited by the presence of non-host hyphae suggesting that these genes play an important role during mycoparasitism. Also, the absence of these corresponding transcripts suggests that the non-host produces

transcription inhibitors. As expected, gene expression of *cytochrome P450* was highly up-regulated early after germination of *S. elegans* conidia. This is in agreement with our finding in the EST data mining study, in which a role in toxin production was assigned to *cytochrome P450*.

RÉSUMÉ

Stachybotrys elegans est un mycoparasite du champignon phytopathogène *Rhizoctonia solani*. L'activité mycoparasitique de *S. elegans* est associée avec la production d'enzymes dégradant la paroi cellulaire telles les chitinases. Cette étude détaille le clonage et la caractérisation d'un ADNc, *sechi44*, qui exprime une endochitinase extracellulaire. Des changements dans l'expression de *sechi44* ont été observés lorsque *S. elegans* interagissait avec son hôte, *Rhizoctonia solani*, et aussi lorsque le mycoparasite poussait sur un medium minimal additionné de différentes sources de carbone et d'azote. Un contact directe avec *R. solani* augmente significativement l'expression de *sechi44* qui décrit un patron d'expression cyclique suggèrant que ce gène n'est pas seulement impliqué dans le mycoparasitisme mais aussi dans la croissance linéaire du mycoparasite. L'addition d'une concentration élevée de glucose et d'ammonium a provoqué une diminution de l'expression de *sechi44*. Ceci suggère que *sechi44* est sujet à la répression par le glucose et l'ammonium. Dans une autre, plusieurs gènes (1016 clones) dont la transcription était substantiellement intensifiée pendant le mycoparasitisme ont été identifiés en utilisant l'hybridation suppressive et soustractive (SSH) et les microarrays. Vingt-cinq pourcents (261 clones) d'entre eux ont été séquencés et des fonctions présumées leur ont été assignées. Parmi eux, 15 séquences partielles exprimées (ESTs) ont été associées à *R. solani* dont leurs fonctions ont été reliées à la défense alors que la majorité des ESTs ont été associées à *S. elegans* et les fonctions présumées ont été reliées au métabolisme des toxines, au procéssus de pathogénicité, à la réponse au stress, à la résistance aux multidrogues, à l'apoptose, au transport, à la synthèse de l'ATP, à la réplication, la transcription et la réparation de l'ADN, à la traduction, à la transduction, à la dégradation des protéines et aux protéines ribosomales. La surexpression de 13 gènes sélectionnés, tous exprimés par *S. elegans*, a été confirmée en utilisant la PCR à transcription inverse quantitatif (QRT-PCR). L'expression temporelle de neuf gènes a été suivie lorsque le mycoparasite poussait sur le mycélium et des sclérotes de *R. solani* (hôte) et *Sclerotinia*

sclerotiorum (non-hôte). L'expression de plusieurs gènes, tels que *seglu*, *selec* et *se151*, était complètement inhibée par la présence d'hyphe du non-hôte suggérant que ces gènes jouent un rôle important lors du mycoparasitisme. Aussi, l'absence de transcrits pour ces gènes suggère que le non-hôte produit un inhibiteur de transcription. Tel que prévu, l'expression génique de *cytochrome P450* est grandement surexprimée tôt après la germination des conidia de *S. elegans*. Ceci appuie les résultats obtenus lors du l'étude d'identification d'ESTs dans laquelle un rôle dans la production de toxines a été assignée pour *cytochrome P450*.

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ABBREVIATIONS

AG	Anastomisis group
BNR	Binucleate *Rhizoctonia* species
cDNA	Complementary deoxyribonucleic acid
CWDE	Cell wall-degrading enzyme
DNA	Deoxyribonucleic acid
DS	Differential screening
EST	Expressed sequenced tag
GFP	Green fluorescence protein
GlcNAc	*N*-acetylglucosamine
gRNA	Genomic ribonucleic acid
GSP	Gene specific primer
HKG	Housekeeping gene
MFS	Major facilitator superfamily
MSM	Minimal synthetic medium
MSMA	Minimal synthetic medium agar
nrDNA	Nuclear ribosomal deoxyribonucleic acid
nrRNA	Nuclear ribosomal ribonucleic acid
OECD	Organization for economic cooperation and development
PCR	Polymerase chain reaction
QRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RACE-PCR	Rapid amplification of cDNA ends-polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SSH	Suppression subtractive hybridization
ST	Subtracted tester
UD	Unsubtracted driver
UT	Unsubtracter tester

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CONTRIBUTIONS OF AUTHORS

This thesis is written in the form of manuscripts according to the "Guidelines Concerning Thesis Preparation". It contains four chapters (3 to 6) representing four separate manuscripts, all of which are either published (chapters 3 and 4), under review (chapter 5), or will be submitted soon (Chapter 6). Each co-author is mentioned, along with his/her corresponding address, at the beginning of each chapter. Below is a general description of the contribution of each co-author. A detailed description is provided in the connecting statements at the beginning of each chapter.

My role in all chapters was to design experimental strategies and conduct and plan all the work of experimental procedures including data mining, data analysis, and the preparation of the first draft of each manuscript. My supervisor, Dr. Suha Jabaji-Hare provided supervision and NSERC funds throughout this study, and secured technical assistance and made every effort to have infrastructure available to me at all steps of this study. She also contributed to the design of experiments, and contributed significantly to the edition and correction of several versions of the manuscripts. Dr. Brian Driscoll, Department of Natural Resource Science, McGill University, provided the phylogeny analysis and reviewed the final version of the manuscript in chapter 3. Dr. Philippe Séguin advised me on the most suitable statistical methods to be applied in Chapters 4 and 6, examined the analyses of data, and reviewed the final versions of the manuscripts in chapters 4 and 6. Drs Roland Brousseau and Luke Masson, BRI, provided substantial help and assistance in microarray experiments and reviewed the final version of the manuscript in chapter 5. Dr. Amélie Dauch provided partial technical assistance to all chapters, in particular in chapter 5, and reviewed the final version of the manuscript of this chapter. Monique Arts conducted the conidial spray and sclerotia experiments, and provided assistance in RNA extraction and cDNA retrotranscription in chapter 6. She also monitored the expression analyses of *sechi44* and calmodulin genes.

CHAPTER 1

1. INTRODUCTION

Fungal phytopathogens cause important damage to crops leading to a decrease of quality and quantity of products, and an increase of cost for producers and consumers (Kubicek, 2004). Chemical fungicides are still intensively used in spite of recent findings demonstrating that some are harmful for human health and the environment and contribute to the build-up of pathogen resistance to pesticides (Dekker, 1976). The fungicides Captan and Ziram are associated with breast cancer and inhibition of human natural killer cell function, respectively (Engel et al., 2005; Taylor et al., 2005), while the fungicide chlorothalonil causes significant decrease in growth rate of the phytoplankton *Dunaliella tertiolecta* (DeLorenzo and Serrano, 2003). Politicians adopted regulations in order to decrease pesticide uses resulting in limited use of fungicides in certain areas (Gerhardson, 2002). Also, the consumers are more aware and conscious of their health and diets, and have the tendency to buy organically-grown produce that are not pesticide treated. All these brought about the idea to decrease the incidence of phytopathogens using biological control.

The antagonistic interactions among microorganisms can be utilized in biological control to reduce diseases caused by plant pathogenic fungi (Lynch, 1990). One form of antagonism that has been extensively studied is mycoparasitism. Most of the research on mycoparasitism has focused on the model biocontrol strains of *Trichoderma* species and much understanding on the role of selected cell wall-degrading enzymes (CWDEs) enzymes and some extracellular proteases and their encoding genes has been gained on these strains (Delgado-Jarana et al., 2002; Grinyer et al., 2005; Pozo et al., 2004; Suarez et al., 2005).

Stachybotrys elegans (Pidopl.) W. Gams is a filamentous fungus commonly found in soil and displays biocontrol capabilities against *Rhizoctonia solani* AG-3 (Benyagoub et al., 1994), a known fungal pathogen of potato. The

mycoparasitic properties of *S. elegans* enable it to colonize its host by accomplishing several successive steps: recognition and production of fimbrial extracellular matrix that surrounds the host cell (Benyagoub et al., 1994; Benyagoub et al., 1996), coiling of the hyphae, and the formation of appressoria that aid in penetrating the host cell wall followed by complete intracellular colonization leading to destruction of hyphae and sclerotia of *R. solani* (Benyagoub et al., 1994). This process is accompanied by the secretion of CWDEs, including chitinases (Taylor et al., 2002; Tweddell et al., 1995), glucanases (Archambault et al., 1998b), and cellulases (Tweddell et al., 1995), which are understood to directly attack the cell of *R. solani*, causing cell wall lysis and death (Benyagoub et al., 1994) making it a good candidate for biological control. Despite these advances, we still have not observed the total diversity of other enzymes and their encoding genes from other mycoparasites, nor do we have comprehensive studies on the regulation of the genetic regulation of these proteins during mycoparasitism. A better understanding of the nature and the regulation of the genes involved in mycoparasitism could improve the performance of mycoparasites as biocontrol agents either by predicting their efficiency in complex soil environments, or by improving formulation for the spread of the mycoparasite. By understanding the basic mechanisms of action and regulation of genes involved in mycoparasitism of *R. solani* by *S. elegans*, the development of approaches for detecting and reducing the impact of *R. solani* or by increasing the biocontrol activity of *S. elegans* may be achieved. This led us to initiate a large-scale EST study using suppression subtractive hybridization (SSH), microarray screening, and real-time QRT-PCR technologies.

1.1. HYPOTHESIS

This study is based on several hypotheses:

(i) Cell wall-degrading enzyme-encoding genes can be cloned based on alignment of similar genes from other mycoparasites including *Trichoderma* species;

(ii) The expression of the endochitinase-encoding gene *sechi44* is altered under different nitrogen and carbon substrates, and during interaction of the mycoparasite *S. elegans* with its host *R. solani*;

(iii) The mycoparasitic relationship between *S. elegans* and *R. solani* elicits the differential expression of the mycoparasite's and host's genes during interaction;

(iv) Gene expression of selected genes, including *sechi44*, is temporally and differentially expressed during interaction of *S. elegans* with live or dead host and non-host.

1.2. OBJECTIVES

Based on our knowledge available on the most studied mycoparasite, *Trichoderma* sp., the global objective of this work was to improve our understanding at the molecular level of the mycoparasitic process, between *S. elegans* and *R. solani*. To reach this general objective, specific objectives were formulated:

(i) To clone and characterize a cell wall hydrolytic enzyme-encoding gene from the mycoparasite *S. elegans*;

(ii) To monitor the temporal gene expression of the endochitinase-encoding gene *sechi44* under different nitrogen and carbon substrates, and during the interaction of the mycoparasite and its host;

(iii) To isolate and characterize hundreds of cDNAs that are differentially expressed during *S. elegans-R. solani* interaction using suppression subtractive hybridization and microarray differential screening;

(iv) To monitor the temporal and differential gene expression of nine genes during the interaction when *S. elegans* is confronted with mycelia and sclerotia of its host *R. solani* as well as with those of the non-host *Sclerotinia sclerotiorum*.

CHAPTER 2

2. LITERATURE REVIEW

2.1. MYCOPARASITISM

Mycoparasites are fungi that parasitize other fungi, their mycelia as well as their vegetative and reproductive structures. There are two forms of mycoparasitism, biotrophic and necrotrophic (Carlile et al., 2001; Cook and Baker, 1983; Perfect and Green, 2001). Biotrophic mycoparasites feed on living hosts in order to have a continuous supply of nutrients (Carlile et al., 2001; Perfect and Green, 2001), and tend to cause only little harm to their host, at least during the early stages of mycoparasitism (Cook and Baker, 1983). In contrast, the necrotrophic mycoparasites invade and kill their host, and use the nutrients that are released from the host (Carlile et al., 2001; Herrera-Estrella and Chet, 2004). The majority of studies on mycoparasitism are conducted on necrotrophic mycoparasites including *Trichoderma* sp. (Kim et al., 2002; Seidl et al., 2005; Viterbo et al., 2002b), *Ampelomyces quisqualis* (Rotem et al., 1999), *Coniothyrium minitans* (Giczey et al., 2001), *Aphanocladium album* (Blaiseau et al., 1992), *Laetisaria arvalis* (Lewis and Papavizas, 1992), *Talaromyces flavus* (Duo-Chuan et al., 2005) and *Stachybotrys elegans* (Archambault et al., 1998b; Benyagoub et al., 1994; Benyagoub et al., 1996; Tweddell et al., 1994; Tweddell et al., 1995).

2.2. ANTAGONISTIC MECHANISMS

The antagonistic mechanisms of mycoparasites include mycoparasitism, antibiosis, competition, induced resistance, tolerance to stress through root and plant development, and inactivation of the pathogen's enzymes (reviewed in Harman, 2000; Herrera-Estrella and Chet, 1998, 2004; Hjeljord and Tronsmo, 1998; Larkin et al., 1998). One or more of these modes of action is employed by a mycoparasite in order to reduce the pathogen's inoculum, prevent or break down the infection, and limit disease development (Elad, 2000; Larkin et al., 1998).

This thesis focuses mainly on the characterization of genes that are expressed during the interaction of the mycoparasite with its host, which are the genes involved in mycoparasitism, antibiosis, and competition.

2.3. *TRICHODERMA* SPP.

The majority of studies conducted on mycoparasites focus on the genus *Trichoderma.* Despite the fact that the mycoparasitic efficiency of strains is linked to growth and environmental conditions (Mathre et al., 1999; Punja and Utkhede, 2004), and to the different mechanisms of action that they apply against their hosts (Krauss and Soberanis, 2001; Talbot Brewer and Larkin, 2005), *Trichoderma* species are considered successful biological control agents.

2.4. MYCOPARASITES AS BIOCONTROL AGENTS

Up to 35 products are registered by various member countries of the Organization for Economic Cooperation and Development (OECD). Among them, five were registered by Canada including Plantshield HC®, Rootshield drench WP®, RootShield® granules, T-22® granules, and Trianum P®. All of them are made from *Trichoderma harzianum* isolate T22 (KRL-AG-2) and are used against the fungal pathogens *Cylindrocladium*, *Fusarium*, *Rhizoctonia*, *Pythium*, and *Thielaviopsis* (Agriculture and Agri-Food Canada, 2005). Even if they are often less efficient than fungicides under field conditions (Benitez et al., 2004; Fravel, 2005; Howell, 2003; Stewart, 2001), their positive characteristics outweigh their performance. These include a narrow-spectrum of target organisms (Larkin et al., 1998), and different mechanisms of action instead of a knockout effect as in the case of fungicides (Gerhardson, 2002). Recent studies had focused to enhance their activity by improving formulation, by changing their physiology, and by using biocontrol agents in combination (Gerhardson, 2002).

2.4.1. Improving formulation

The formulation is an important part in the development of biocontrol agent systems. Where, how, when, how much, which state and form a biocontrol

agent is applied is determined by the type of formulation (Larkin et al., 1998). The weakest characteristic of biocontrol formulation, apart from the consistency and the effectiveness, is the length of shelf life (Spadaro and Gullino, 2005). In order to improve formulation and increase the efficiency of biocontrol agents, their different mechanisms of action have to be understood. To reach this goal, genetic studies of different mycoparasites interacting with their hosts are imperative.

2.4.2. Changing the physiology of mycoparasites

The genome of mycoparasites represents a potential source of powerful antifungal genes that could be used for improving mycoparasite's efficiency (Whipps, 2001). By introducing several copies of an endogene or an exogene into a mycoparasite, its physiology can be changed in such a way that its antagonistic activity against its host is increased. For example, introduction of multiple copies of the protease encoding gene *prb1* in *T. atroviride* triggered a better efficiency in the control of *Rhizoctonia solani* (Flores et al., 1997) while overexpression of the endo-β-1,4-glucanase *egl1* in *T. longibrachiatum* increased its biocontrol activity against *Pythium ultimum* (Migheli et al., 1998).

2.4.3. Combining different biocontrol agents and integrated pest managment

Combining different antagonists or biocontrol methods allows a synergetic effect by triggering a wider spectrum of control (Spadaro and Gullino, 2005) and may overcome the problem of variation due to different environmental factors such as humidity, temperature, and pH (Guetsky et al., 2001; Larkin et al., 1998; Talbot Brewer and Larkin, 2005; Whipps, 2001). It has been shown that combination of different but compatible strains of biocontrol agents can generally improve the efficiency of control (Hwang and Benson, 2002; Krauss and Soberanis, 2001; Talbot Brewer and Larkin, 2005) such as in the case of the mycoparasite *Clonostachys rosea*. Five different strains of *C. rosea* improved the control of two important diseases in cocoa pods, *Moniliophtora roreri* (causing moniliasis) and *Crinipellis perniciosa* (causing witches' brooms), and increased the percentage of healthy pods (Krauss and Soberanis, 2001). Furthermore, it has

been established that using antagonistic fungi in an integrated pest management could improve the efficiency of the mycoparasite and reduce the amount of chemical that are applied. For example, the use of mycoparasites, such as *T. atroviride*, *T. harzianum*, *L. arvalis*, and *A. quisqualis,* with fungicide applications increased the efficacy of pathogen control of several fungi including *R. solani* and *Fusarium* spp. compared when the two treatments were applied alone, and reduced the number of fungicide applications. (Chet and Inbar, 1994; Conway et al., 1997; Harman, 2000; Howell et al., 1997; Lorito et al., 1998; Shishkoff and McGrath, 2002; Spadaro and Gullino, 2005; Wang et al., 2005).

2.5. MECHANISM OF ACTIONS DURING MYCOPARASITISM

Mycoparasitism is a complex process that is separated into several successive steps including (i) chemotropic growth towards the host, (ii) host recognition and attachment, (iii) secretion of several cell wall-degrading enzymes (CWDEs) by the mycoparasite, (iv) penetration and colonization of the host hyphae, and (v) lysis of the host hyphae (Herrera-Estrella and Chet, 2004; Whipps, 2001). These steps have been comprehensibly reviewed by Benitez et al. (2004), Chet et al. (1998), Herrera-Estrella and Chet (1998, 2004).

2.5.1. Chemotropic growth

The mycoparasite grows toward its host in response to a stimuli or a gradient of chemicals produced by the host (Chet et al., 1998; Chet et al., 1981; Herrera-Estrella and Chet, 2004). Neither the nature nor the host's specificity of this signal is yet identified. Apparently, mycoparasites constitutively produce exochitinases which trigger the release of cell wall oligomers from the host which in turn induce the expression of endochitinases even before the contact occurs between the mycoparasite and the host (Brunner et al., 2003a; Viterbo et al., 2002a; Zeilinger et al., 1999).

2.5.2. Host recognition and attachment

When the mycoparasite reaches its host, its hyphae coil around the host's hyphae probably in response to lectins present at the surface of the host's hyphae (Barak and Chet, 1990; Barak et al., 1985; Elad et al., 1983a; Inbar and Chet, 1996), followed by their attachment to the host's hyphae by means of appressoria developed by the mycoparasite (Benyagoub et al., 1994; Inbar and Chet, 1992; Inbar and Chet, 1994). In some cases, the mycoparasite produces in addition to infection pegs, a fimbrial extracellular matrix that surrounds the host cell (Benyagoub et al., 1994; Benyagoub et al., 1996; Chet et al., 1981).

2.5.2.1. Genes encoding recognition of the host

Lectins are glycoproteins that are produced at the surface of host's hyphae, and bind to specific galactose and fucose residues present on the cell wall of mycoparasites (Barak et al., 1985; Elad et al., 1983a). Lectins from *R. solani* and *Sclerotium rolfsii* have been purified and characterized (Barak et al., 1985; Inbar and Chet, 1994), but corresponding lectin genes from the mycoparasite have not been cloned. However, the closest gene from a mycoparasite that may play a part in recognition is the G-protein α-subunit encoding gene *tga1* from *T. virens* which was found to be associated with coiling and conidiation (Rocha-Ramirez et al., 2002).

2.5.3. Secretion of CWDEs and penetration of the host hyphae

Once the interaction between the mycoparasite and its host has begun, the mycoparasite penetrates the rigid host cell wall by secreting cell wall lytic enzymes referred to as CWDEs whose role is to degrade the hyphae cell wall (Chet et al., 1998). The most studied CWDEs are chitinases, glucanases, lipases, and proteases, (Kubicek, 2004), and several of their proteins and genes have been characterized from different mycoparasites.

2.5.4. Genes encoding cell wall-degrading enzymes

The cell wall of eumycotan fungi is composed mainly of various macromolecules of polysaccharides and lipids. β-glucan, chitin, and mannoproteins (glycoproteins) are macromolecules responsible for the strength and the shape of the cell wall (Ruiz-Herrera, 1992) making them an interesting target of attack for biocontrol agents.

The antifungal proteins most frequently described are chitinases and β-1,3-glucanases. These enzymes catalyze the hydrolysis of chitin and β-1,3-glucan, respectively, both major components of the cell walls of higher fungi (Wessels and Sietsma, 1981). Both chitinases and glucanases have several functions in fungi (Adams, 2004; Duo-Chuan, 2006): i) a nutritional role in saprotrophism and energy-source exhaustion, ii) a physiological role in morphogenetic processes during fungal development and differentiation (Fleet and Phaff, 1981; Kamada, 1994), iii) a possible role in protecting cells against dehydration (Halliwell, 1979; Wessels and Sietsma, 1981), and iv) an ecological role where the mycoparasite's chitinases and β-1,3-glucanases are involved in establishing and maintaining a relationship with its host (Benhamou and Chet, 1997; Gooday, 1990; Thrane et al., 1997). Less well characterized, but also involved in the mycoparasitic process, are proteases, lipases and lyases (Benitez et al., 1998). It has been suggested that a basal level of CWDEs are produced resulting in the release of inducing polysaccharides from the host cell which in turn stimulate the production of different CWDEs by the mycoparasite increasing its antagonistic activity (Steyaert et al., 2003).

The CWDE-encoding genes of biocontrol fungi represent an alternative to the corresponding plant chitinase and glucanase genes because their products: i) are highly effective against a broad range of pathogens including *Rhizoctonia, Fusarium, Alternaria, Ustilago, Venturia, Pythium, Phytophthora* and *Botrytis* species (Di Pietro et al., 1993; Lorito, 1998; Lorito et al., 1993; Lorito et al., 1994a; Lorito et al., 1994b); ii) are able to degrade not only the immature cell wall at the fungal apex, but also the strong chitin-glucan complexes of the mature cell walls and sclerotia (Benyagoub et al., 1994; Lorito et al., 1993); iii) may be

overproduced *in planta* with positive effects on disease resistance (Bolar et al., 2001; Bolar et al., 2000; Emani et al., 2003; Lorito, 1998; Mora and Earle, 2001b); and iv) have a biological activity which can be synergistically enhanced in combination with other CWDEs, antibiotics, plant pathogenesis related (PR) proteins, or azole fungicides (Lorito et al., 1994b; Mora and Earle, 2001a; Schirmbock et al., 1994).

Most of the world's research on mycoparasites has focused on a few model biocontrol strains such as *T. harzianum*, *T. hamatum,* and *T. atroviride*, and much understanding on the role of selected cell wall-degrading enzymes, especially chitinases and to a lesser extent glucanases and proteases, has been gained on these strains (Carsolio et al., 1999; Elad and Kapat, 1999; Garcia et al., 1994; Mach et al., 1999; Ramot et al., 2000; Zeilinger et al., 1999). Despite these advances, we still have not observed the total diversity of cell wall hydrolases produced by fungal mycoparasites nor have we gained substantial information on the regulation of the genes that encode them (Donzelli and Harman, 2001).

2.5.4.1. Chitinases

Chitinases are enzymes capable of hydrolyzing chitin to its monomer *N*-acetylglucosamine (GlcNAc). Chitinases hydrolyze the β-1,4-linkages of chitin and are broadly classified as endochitinases and exochitinases. The endochitinase (EC 3.2.1.14) cleaves at internal points randomly in the chitin chain generating soluble low molecular mass multimers of GlcNAc. The exochitinases can be divided into two subcategories : 1) chitobiosidase (EC 3.2.1.29) which cleaves at the non-reducing ends of chitin with the release of successive diacetylchitobiase units and 2) 1,4-β-*N*-acetylglucosaminidase (or chitobiase) (EC 3.2.1.30) which cleave the oligomeric products of endochitinases and chitibiosidases generating monomers of GlcNAc (Duo-Chuan, 2006; Lorito et al., 1998).

2.5.4.1.1. Chitinase-encoding genes and their role

Several chitinase-encoding genes have been cloned to date (Table 2.1). Apart from the ones cloned from the mycoparasites *Coniothyrium minitans*

Table 2.1. Chitinase-encoding genes of mycoparasites.

Gene	Classification	Enzyme	Weight (kDa)	species	Reference
chi1	endo		42	*Coniothyrium minitans*	Giczey and Hornok, unpublished
	endo		42	*Aphanocladium album*	Blaiseau et al., 1992
chit42	endo	CHIT42	42	*T. harzianum* CECT2413	Garcia et al., 1994
chit42	Endo	CHIT42	42	*T. hamatum*	Steyaert et al., 2004
ech42	endo	ECH42	42	*T. atroviride* IMI206040	Carsolio et al., 1994
cht42	endo		42	*T. virens* tv29-8	Beak et al,. 1999
ThEn42	endo		42	*T. atroviride* P1	Hayes et al., 1994
chi18-2	endo		42	*T. reesei*	Seidl et al., 2005
chi18-3	endo		42	*T. reesei*	Seidl et al., 2005
chi18-4	endo		42	*T. reesei*	Seidl et al., 2005
ech2	endo		42	*T. virens* tv29-8	Kim et al., 2002
ech3	endo		42	*T. virens* tv29-8	Kim et al., 2002
ech30	endo		30	*T. atroviride*	Klemsdal et al., 2006
chit33	endo	CHIT33	33	*T. harzianum* CECT2413	Limon et al., 1995
chit36	endo	CHIT36	36	*T. harzianum* TM	Viterbo et al., 2001
chit36	endo	CHIT36	36	*T. asperellum* T-203	Viterbo et al., 2002
				T. atroviride P1	
chit37	endo	CHIT37	37	*T. harzianum* CECT2413	Viterbo et al., 2002
				T. inhamatum CECT2424	
chit46	endo		46	*T. reesei*	Ike et al., 2005
Th-Ch	endo		42	*T. harzianum* Tam-61	Fekete et al., 1996
ENC1	endo		42	*T. harzianum* T25-1	Draborg et al, 1996
cht1	endo		33	*T. virens* tv29-8	Kim et al., 2002
cht2	endo		33	*T. virens* tv29-8	Kim et al., 2002
chit18-10	endo			*T. reesei*	Seidl et al., 2005
chit18-13	endo			*T. reesei*	Seidl et al., 2005
exc1	exo	EXC1	73	*T. harzianum* T25	Draborg et al., 1995
exc2	exo	EXC2	73	*T. harzianum* T25	Draborg et al., 1995
nag1	exo	NAG1	72	*T. atroviride* P1	Peterbauer et al., 1996
nag1	exo			*T. virens* tv29-8	Kim et al., 2002
nag2	exo			*T. virens* tv29-8	Kim et al., 2002

*Other 42-kDa endochitinase-encoding genes have been cloned in several mycoparasitic and non-mycoparasitic species of *Trichoderma* and a list of several 42-kDa-encoding genes from different *Trichoderma* species are published by (Lieckfeldt et al., 2000).

(Giczey et al., unplublished), *A. album* (Blaiseau et al., 1992), and *S. elegans* (Morissette et al., 2003), all the others are reported from different species of the genus *Trichoderma*. The majority of these genes encode for endochitinases including the most studied one, the 42-kDa-encoding gene (Table 2.1). Several studies have been conducted on the 42-kDa endochitinase gene which is not an exclusive property of the mycoparasite strains even though the 42-kDa chitinase plays a major role in mycoparasitism (Garcia et al., 1994). This suggests that the 42-kDa chitinase plays not only a role in mycoparasitism but also in fungal development and differentiation (Fleet and Phaff, 1981; Kamada, 1994). In the mycoparasitic process, *ech42* is the only chitinase to be expressed before contact of *T. atroviride* with *R. solani* indicating that this enzyme could be involved in the very early stages (i.e., between recognition and contact) of the process (Zeilinger et al., 1999). The expression of this gene is induced by a diffusible factor produced by the host (Cortes et al., 1998). In another study, the 42-kDa chitinase was highly expressed when *Trichoderma* strains were grown on chitin-supplemented medium or in dual cultures with a host (Chet et al., 1998; Geremia et al., 1993). Furthermore, transgenic plants containing the 42-kDa chitinase showed a higher resistance to several plant pathogenic fungi (Bolar et al., 2000; Emani et al., 2003; Lorito, 1998; Mora and Earle, 2001b). These results suggest that this gene is directly involved in mycoparasitism. An increase in the production of *ech42* by transgenic *Trichoderma* strains does not affect the mycoparasitic activity, which suggests that the level of chitinases naturally secreted by *Trichoderma* spp. is high enough for efficient biocontrol of the phytopathogenic fungi (Carsolio et al., 1999).

The regulation of the remaining chitinases and their-encoding genes has not been studied to the same degree as *ech42* and its corresponding protein, with very limited available information. Generally, the expression of chitinases increases in the presence of host cell wall and chitin and is repressed by high concentrations of glucose and nitrogen (Dana et al., 2001; Donzelli and Harman, 2001; Kim et al., 2002; Viterbo et al., 2002a). Similar to *ech42,* the expression of *chit36* is induced by soluble factors released by the host. Overexpression of *chit36*

in *T. harzianum* increased the rate of growth inhibition of *Fusarium* and *Sclerotinia rolfsii* on agar plates, although, no difference in the ability to control the plant pathogens was observed. This result suggests that the amount of the naturally secreted enzyme *chit36* is sufficient to control its hosts (Viterbo et al., 2001). Similarly, the overexpression of *chit33* led to a higher antagonistic activity against *R. solani* and increased the efficacy of *T. harzianum* to inhibit the host's growth (Limon et al., 1999).

2.5.4.2. Glucanases

Glucanases involved in mycoparasitism are classified according to their mechanism of action. These enzymes can hydrolyze α- or β-linkage, and 1,3-, 1,4-, or 1,6-linkage. Based on their hydrolytic action, glucanases from mycoparasites are divided into 4 classes: 1) β-1,3-glucanases are enzymes capable to hydrolye the 1,3-linkages in 1,3-β-D-glucan. β-1,3-glucanases can have two mechanisms of action : a) exo-β-1,3-glucanases (EC 3.2.1.58; 1,3-β-D-Glucan glucanohydrolase) which hydrolyze the 1,3-β-glucan chain by sequentially cleaving glucose residues from the non-reducing end. Consequently, the sole hydrolysis product is usually glucose, and b) endo-β-1,3-glucanases (or endo-1,3-β-glucosidase) (EC 3.2.1.39; 1,3-β-D-Glucan glucanohydrolase) which cleaves β-1,3-linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides. Two hydrolysis products can be released that categorize endo-β-1,3-glucanases into two groups: a) those principally releasing oligosaccharides during hydrolysis, and b) those rapidly producing glucose and polysaccharides (Benitez et al., 1998); 2) β-1,4-glucanases (cellulases) which cleave the cellulose have three mechanisms of action a) exo-cellulases (EC 3.2.1.91, β-1,4-D-glucan cellobiohydrolases) which cleave cellobiose unit from the ends of cellulose and its oligomers; b) endo-β-1,4-glucanase (EC 3.2.1.4) which cleave β-1,4-linkages at random sites along the cellulose (polysaccharide chain), releasing smaller oligosaccharides; and c) β-1,4-glucosidases (EC 3.2.1.21) which cleave cellobiose units to glucose. 3) endo-β-1,6-glucanases (EC3.2.1.75) which are specific to β-

1,6-linkages with an endo activity; and 4) endo-α-1,3-glucanases (EC 3.2.1.59, laminarinases) catalyze the hydrolysis of α-1,3-linkages.

2.5.4.2.1. Glucanase-encoding genes and their role

Genes encoding all of these classes have been cloned and characterized (Table 2.2). Apart from the endo-β-1,3-glucanase-encoding genes from the mycoparasites *A. quisqualis* (Rotem et al., 1999) and *C. minitans* (Giczey et al., 2001), all the others are reported from *Trichoderma* spp.. Generally, the expression of glucanase-encoding genes is induced by the host, the host's cell wall, and laminarin and repressed by glucose and primary nitrogen sources (Ait-Lahsen et al., 2001; Cohen-Kupiec et al., 1999; de la Cruz et al., 1995b; Donzelli et al., 2001; Kaur et al., 2005; Kumar et al., 2000; Nobe et al., 2004; Sanz et al., 2005; Vazquez-Garciduenas et al., 1998). In the case of β-1,3-glucanases, their production appeared to be dependant on the amount of β-1,3-glucan in the host cell wall (Vazquez-Garciduenas et al., 1998). The inhibition of their expression by high levels of glucose and nitrogen suggests that the glucanase-encoding genes are under catabolite repression (Ait-Lahsen et al., 2001; Bara et al., 2003; Donzelli and Harman, 2001; Kaur et al., 2005).

β-1,3-glucanases, α-1,3-glucanases, and β-1,6-glucanases inhibit spore germination and the growth of the host (Ait-Lahsen et al., 2001; Benitez et al., 2004; de la Cruz et al., 1995a; de la Cruz et al., 1995b; Sun et al., 2006). In addition to β-1,3-glucanases, β-1,4-glucanases play an important role in the degradation of the host's cell wall in the mycoparasitism of lower fungi such as *Pythium* since cellulose is abundant in their cell wall (Herrera-Estrella and Chet, 2004).

2.5.4.3. Proteases

Proteases (or peptidases or proteolytic enzymes or peptide hydrolases) are enzymes that hydrolyze peptide bonds (CO-NH) of proteins to free amino acids (Monod et al., 2002). They are classified following their mode of action and their

Table 2.2. Glucanase-encoding genes of mycoparasites.

Gene	Classification	Enzyme	Weight (kDa)	Species	Reference
exgA	endo β,1-3	EXGA	84	*Ampelomyces quisqualis*	Rotem et al., 1999
cmg1	exo β,1-3	CMG1	82	*Coniothyrium minitans*	Giczey et al., 2001
bgn13.1	endo β,1-3	BGN13.1	78	*T. harzianum* CECT2413	De la Cruz et al., 1995
gluc78	exo β,1-3	GLUC78	78	*T. atroviride* P1	Donzelli et al., 2001
bgn1	endo β,1-3		78	*T. virens* tv29-8	Kim et al., 2002
bgn2	endo β,1-3		78	*T. virens* tv29-8	Kim et al., 2002
lam1.3	exo β,1-3	LAM1.3	110	*T. harzianum* T-Y	Cohen-Cupiek et al., 1999
lam1.3	exo β,1-3	LAM1.3	110	*T. hamatum*	Steyaert et al., 2004
lamA1	endo β,1-3	LAMAI	76	*T. viride*	Nobe et al., 2004
cbh1	exo β,1-4	CBHI		*T. reesei*	Shoemaker et al., 1983
cbh1	exo β,1-4			*T. viride*	Teeri et al., 1983
cbh1	exo β,1-4	CBHI		*T. koningii* G-39	Wey et al., 1994
cbh1	exo β,1-4			*T. harzianum*	Guilfoile et al., 1999
cbh2	exo β,1-4	CBHII		*T. reesei*	Chet et al., 1987
cbh2	exo β,1-4	CBHII		*T. koningii*	Teeri et al., 1987
egl1	endo β,1-4	EGI	50	*T. reesei* RUT C-30	Pentilla et al., 1986
egl1	endo β,1-4			*T. longibrachiatum*	Van Arsdell et al., 1987
egl2	endo β,1-4			*T. viride*	Saloheimo et al., 1988
egl3	endo β,1-4	EGIII	44	*T. viride*	Liu et al., 2004
egl4	endo β,1-4	EGIV		*T. reesei*	Saloheimo et al., 1997
egl5	endo β,1-4	EGV		*T. reesei*	Saloheimo et al., 1994
bgl1	β,1-4 glucosidase	BGL1		*T. reesei*	Mach, 1993
bgl2	β,1-4 glucosidase	BGL2	52	*T. reesei*	Takasima et al., 1999
bgn16.2	endo β,1-6	BGN16.2	43	*T. harzianum* CECT2413	Lora et al., 1995
bgn3	endo β,1-6			*T. virens* tv29-8	Kim et al., 2002
agn13.1	endo α,1-3	AGN13.1	75	*T. harzianum* CECT2413	Ait-Lahsen et al., 2001
agn13.2	endo α,1-3	AGN13.2	75	*T. asperellum* CECT20539	Sanz et al., 2005
	endo α,1-3		72	*T. harzianum* Rifai	Fuglsang et al., 2000

active sites such as aspartic, serine, cystein, threonin, and metallo proteases (Monod et al., 2002). Based on studies on extracellular proteases from *Trichoderma* spp., serine proteases are related to biocontrol processes (Flores et al., 1997; Grinyer et al., 2005; Hanson and Howell, 2004; Pozo et al., 2004; Suarez et al., 2005), while aspartic proteases are associated with the degradation of proteins (Delgado-Jarana et al., 2002; Haab et al., 1990; Margolles-Clark et al., 1996).

2.5.4.3.1. Protease-encoding genes and their role

Seven protease-encoding genes from mycoparasites have been cloned. All belong to *Trichoderma* spp. (Table 2.3) and are now considered potential virulence factors (Monod et al., 2002). These enzymes inactivate hydrolytic enzymes produced by *Botrytis cinerea* on bean leaves and inhibit spore germination of the host and maturation of its enzymes (Elad and Kapat, 1999; Howell, 2003). The expression of *prb1,* the most studied protease-encoding gene, is induced by chitin, autoclaved mycelia, and fungal cell wall preparation (Geremia et al., 1993), and gene regulation is dependant on cell wall composition of the host (Pozo et al., 2004). Similar to chitinases, serine protease-encoding genes are repressed by glucose, glycerol, and exogenous nitrogen sources such as yeast extract suggesting that their expression is under carbon and nitrogen catabolite repression (Cortes et al., 1998; Geremia et al., 1993; Olmedo-Monfil et al., 2002; Steyaert et al., 2004). Mycoparasite/host studies demonstrated that the expression of *prb1* is induced during the interaction of *T. hamatum/S. sclerotiorum* and *T. atroviride/R. solani* (Carsolio et al., 1994; Cortes et al., 1998; Steyaert et al., 2004), even before physical contact for the latter interaction (Flores et al., 1997), and by diffusible factors produced by the host (Cortes et al., 1998). Contrary to serine proteases, the regulation of the aspartic protease-encoding gene *pepA* is induced by yeast extract (Delgado-Jarana et al., 2002; Viterbo et al., 2004), and prior to physical contact with *R. solani*, whereas the regulation of *pepB* is not affected by the presence of *R. solani* (Viterbo et al., 2004). Similar to *prb1*, the expression induction of the aspartic protease *P6281* from *T. harzianum* occurs

Table 2.3. Protease-encoding genes of mycoparasites.

Gene	Classification	MEROPS ID	Enzyme	Weight (kDa)	Species	Reference
Prb1	Serine protease	S08.066		31	*T. hamatum*	Steyaert et al., 2004
Prb1	Serine protease	S08.066		31	*T. atroviride*	Geremia et al. 1993
Tvsp1	Serine protease	S08.066			*T. virens*	Pozo et al., 2004
Pra1	Trypsin protease	S01.103			*T. asperellum*	Suarez et al., 2004
papA	Aspartic protease	A01.027			*T. asperellum*	Delgado-Jarane et al., 2002
papB	Aspartic protease	A01.027			*T. asperellum*	Viterbo et al., 2004
P6281	Aspartic protease	A01.027	P6281	33	*T. harzianum*	Suarez et al., 2005

very early suggesting that this gene is involved in early stages of the mycoparasitic processes (Suarez et al., 2005).

2.5.4.4. Increasing resistance and improving biocontrol efficacy with CWDEs

CWDE-encoding genes can be used either to improve plant resistance against phytopathogens or increase the biocontrol activity of mycoparasites. So far, different plants and mycoparasites have been transformed with these genes successfully. For example, transgenic potato and tomato plants, cotton, and apple trees carrying a 42-kDa endochitinase-encoding gene of *Trichoderma* spp. were highly tolerant or completely resistant to *Alternaria alternata*, *A. solani, B. cinerea*, and *R. solani* (Emani et al., 2003; Lorito et al., 1998), and to *Venturia inaequalis* (Bolar et al., 2000). Also, introduction of multiple copies of the encoding protease gene *prb1* in *T. atroviride* imporved efficiency of control of *R. solani* on cotton plants (Flores et al., 1997) while overexpression of the endo-β-1,4-glucanase encoding gene *egl1* in *T. longibrachiatum* increased its biocontrol activity against *Pythium ultimum* on cucumber (Migheli et al., 1998).

Lorito et al. (1994b) showed that there is a synergistic action between CWDEs suggesting that a combination of genes might improve the biocontrol activity. Recently, Bolar et al. (2001) introduced into four-week-old apple seedlings an endochitinase- (*ech42*) and an exochitinase- (*nag1*) encoding genes of *T. atroviride*. Transgenic plants expressing both genes were more tolerant to *V. inaequalis* than transgenic plants expressing only one of them.

However, the introduction of foreign genes, either in plants or mycoparasites, does not always guarantee positive effects. For example, the transformation of young apple plants with *ech42* had a negative effect on the growth of the plant (Bolar et al., 2000). Equally, transgenic *Trichoderma* species having multiple copies of *prb1* demonstrated a poor growth rate, possibly because over-production of proteases has a toxic effect on the mycoparasite (Flores et al., 1997).

2.5.5. Lysis of the host hyphae

Following the degradation of the host's cell wall, the mycoparasite grows inside the host's hyphae which become less dense and disorganized (Benyagoub et al., 1994). In response to the invasion of the mycoparasite, the host's cells produce a sheath matrix, named papillae, which engulfs the mycoparasite's hyphae possibly in order to discourage the growth of the mycoparasite (Benyagoub et al., 1994; Elad et al., 1983b).

2.5.6. Antibiosis

Inhibition or destruction of a pathogen by secondary metabolites of an antagonist is referred to as antibiosis. The secondary metabolites could be toxins and antibiotics, and in some instances, enzymes are included in antibiosis (Larkin et al., 1998). Mycoparasites such as *Trichoderma* produce several substances having antibiotic activity (Sivasithamparam and Ghisalberti, 1998). Among these substances are alkyl pyrones, isonitriles, polyketides, peptaibols, diketopiperazines, sesquiterpenes, and steroids (Harman, 2000; Howell, 1998). Antibiosis appears to be an important mode of action for *Trichoderma* species (Di Pietro et al., 1993; Lorito et al., 1996a; Lorito et al., 1996c; Schirmbock et al., 1994).

2.5.6.1. Genes encoding antibiotics and toxins

Peptaibols are linear oligopeptides forming voltage-gated ion channels in lipid cell membranes. They act in synergy with CWDEs by triggering a modification in the membrane permeability of liposomes in the absence of applied voltage. While CWDEs degrade the host's hyphae, peptaibols impair the host's ability to repair this damage by inhibiting the activity of chitin and β-glucan synthases (Kubicek, 2004). Recently, three genes encoding peptaibol synthetase *tex (T. virens)*, *salps1 (T. harzianum)* and *pes* (*T. asperellum*) have been cloned (Chutrakul and Peberdy, 2005; Vizcaino et al., 2005; Wiest et al., 2002).

2.5.7. Competition

Microorganisms usually compete for nutrients (carbon, nitrogen, iron, vitamins as thiamine) and space. Antagonists including non-pathogenic *Fusarium* species (Fravel et al., 2003), *Trichoderma* species (Hjeljord and Tronsmo, 1998), and non-pathogenic binucleate *Rhizoctonia* species (Herr, 1995) can be more efficient than pathogens in utilizing nutrients and competing for space. One such mechanism that helps antagonists utilize nutrients more efficiently is the production of siderophores. Siderophores are an important mode of competition for iron in the rhizosphere (Loper and Buyer, 1991; Wilhite et al., 2001), and several pathogens employ them for iron acquisition in order to limit the supply of ion to their host. Since iron is essential for metabolism and growth, limited availability of this ion is detrimental for the host (Zarnowski and Woods, 2005). It has been shown that *T. harzianum* is an aggressive competitor against several soilborne plant pathogens due to its ability to produce siderophores chelating metals such as Zn, Cu, and Fe (Altomare et al., 1999; Chet and Inbar, 1994).

2.5.7.1. Genes involved in competition

Two genes that might be involved in competition between mycoparasites and their hosts have been cloned. One of them, *Psy1* cloned from *T. virens*, seems to be related to the production of siderophores (Wilhite et al., 2001). The second is a high-affinity glucose transporter, *Gtt1*, from *T. harzianum* (Delgado-Jarana et al., 2003). *psy1* encodes a segment of a peptide synthase which is involved in the biosynthesis of antibiotics as well as siderophores for iron uptake (Wilhite et al., 2001). The loss of expression of *psy1* in *T. virens* resulted in a poor growth rate in low-iron medium, incapacity for iron acquisition, and a low amount of siderophores, suggesting that *psy1* is involved in biosynthesis of siderophores (Loper and Buyer, 1991; Wilhite et al., 2001). The knock out of this gene does not affect the biocontrol activity of *T. virens* against *P. ultimum* and *R. solani* (Wilhite et al., 2001).

The gene *gtt1* encodes a high-affinity glucose transporter. The expression of *gtt1* is strongly induced by carbon starvation and repressed by high

concentration of glucose suggesting that this gene is under carbon catabolite repression. During interaction studies of *T. harzianum* with *R. solani*, *gtt1* was shown to be overexpressed. It is believed that the overexpression of this gene increases the velocity of glucose transport and enables rapid intake of glucose (Delgado-Jarana et al., 2003). This rapid intake could help to compete for nutrients during mycoparasitic interactions (Delgado-Jarana et al., 2003).

2.5.8. Other genes involved during mycoparasitism

Other genes related to mycoparasitism have also been cloned from *Trichoderma* species. These genes are G-protein α-subunits (Gα-subunit) from *T. atroviride* (*tgaA, tgaB, tga1* and *tga3*) (Mukherjee et al., 2004; Omero et al., 1999; Rocha-Ramirez et al., 2002) and the MAP kinases *tvk1* and *tmkA* from *T. virens* (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003).

G-proteins are composed of three subunits, α, β, and γ. This heterotrimeric protein is involved in signal transduction from a receptor to an intracellular target (Reithner et al., 2005; Rocha-Ramirez et al., 2002). Gα-subunits have multiple roles in several fungal pathogens, including the recognition process, virulence, formation of infection structures, antibiotic production, and secretion of extracellular enzymes (Benitez et al., 2004; Boelker, 1998; Horwitz et al., 1999; Reithner et al., 2005; Rocha-Ramirez et al., 2002; Steyaert et al., 2003; Zeilinger et al., 2005). In order to determine the role of Gα-subunits in mycoparasitism, mutants and knockout lines were developed. Based on sequence studies, three major subgroups were identified (Boelker, 1998). Group I includes *tga1* from *T. atroviride* and *tgaA* from *T. virens*. Over-production of *tga1* stimulated coiling around the host, improved the capacity to overgrow the host, and inhibited conidiation of the mycoparasite, whereas the knockout of this gene produced the opposite effect and reduced the expression of chitinases and the production of antifungal sesquiterpene-type secondary metabolites (Reithner et al., 2005; Rocha-Ramirez et al., 2002). These results confirm that *tga1* is involved in conidiation and coiling, the production of chitinases and antifungal metabolites, as well as in vegetative growth (Reithner et al., 2005). Interestingly, even if

overgrowth is absent in *tga1* knock out lines, growth of the host was still inhibited as soon as both mycelia were within by 1-2 cm, suggesting that inhibitory substances are secreted by the mycoparasite (Reithner et al., 2005). On the other hand, knock out experiments involving the homologous gene *tgaA* from *T. virens* did not affect coiling or conidiation (Mukherjee et al., 2004). The above findings suggest that Gα-subunits of subgroup I have different functions during mycoparasitism (Zeilinger et al., 2005). Subgroup II includes the gene *tgaB* from *T. virens* which does not show any specific role in mycoparasitism (Mukherjee et al., 2004). Subgroup III includes the gene *tga3* from *T. atroviride,* and this gene plays an important role in mycoparasitism as confirmed by knock out experiments. Transformants lacking *tga3* not only lost their virulence against *R. solani*, *B. cinerea*, and *S. sclerotiorum* (Zeilinger et al., 2005), but exhibited a reduction and delay in conidial germination and the absence of mycoparasitism-related infection structures, leading to loss of mycoparasitism, and a decrease in the expression of the extracellular CWDEs encoded by *nag1* and *ech24* (Zeilinger et al., 2005).

Two genes encoding for mitogen-activated protein kinases (MAPK), *tmkA* and *tvk1*, were cloned from *Trichoderma* species. These genes are involved in the process of biocontrol by transducing signals by means of a protein phosphorylation cascade (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003). The gene *tmkA* seems to be important for mycoparasitism since knock out of this gene triggered a loss of virulence in a host-specific manner. The mutants parasitized hyphae of *R. solani* but not of *S. rolfsii* (Mukherjee et al., 2003). This gene is also responsible for repression of conidiation in the dark and possibly in activation of transcription factors for genes encoding CWDEs (Mukherjee et al., 2003). Similarly, the gene *tvk1* represses conidiation, growth rate, and pigmentation. Contrary to *tmkA*, the null mutant *tvk1* has a greater capacity to control *R. solani* and *S. rolfsii,* suggesting that *tvk1* plays a major role in the regulation of hydrolytic enzyme-encoding genes (Mendoza-Mendoza et al., 2003).

Recently, a global analysis of gene expression in mycelium of *T. harzianum* has been published using an expressed sequence tag (EST) approach

(Liu and Yang, 2005). However, the analysis of the identified clones did not reflect the genetic regulation of the mycoparasitic interaction with a living host and its defense reaction, but was rather the result of gene expression in the presence of cell wall preparation. Thus, it is not surprising that among the genes identified, several were related to the hydrolysis of fungal cell walls.

The identification of genes presumably involved in the mycoparasite-host interaction was recently analyzed. Carpenter et al. (2005) applied suppression subtractive hybridization (SSH) and explored differential gene expression of the mycoparasite *T. harzianum* in the presence of the living host *Sclerotinia sclerotiorum*. Unfortunately, only a limited subset of novel genes was identified (a total of 19) resulting in an incomplete view of genetic regulation during mycoparasitism. Except for this recent finding, differential expression studies on mycoparasites other than *Trichoderma* in the presence of live hosts have not been reported.

2.6. *RHIZOCTONIA SOLANI*

Rhizoctonia solani Kühn is the anamorph of *Thanatephorus cucmeris* (Frank) Donk. This fungus belongs to the phylum Basidiomycota, the class Homobasidiomycetes, the order Ceratobasidiales, and the family Ceratobasidiaceae. *R. solani* is a species complex (Gonzalez Garcia et al., 2006). It is a destructive pathogen against more than 200 species, including several important crops such as rice, corn, wheat, soybeans, and potato (Gonzalez et al., 2006). *R. solani* causes important damage leading to damping-off of seedlings, root, crown and stem rots, and sheath blight of cereals (Anderson, 1982; Ogoshi, 1987). Its strains have been divided into anastomosis groups (AGs) based on the ability of their hyphae to fuse, which is a common method for gene exchange in fungi. Isolates belonging to the same AG can recognize and fuse only with each other (Parmeter et al., 1969). Currently, there exists 14 AGs and 31 subgroups exist (Carling et al., 2002).The hyphae of isolates belonging to AG-1 to AG-13 fuse only among themselves, while isolates belonging to AG-BI (bridging isolates) are capable of fusing hyphae among themselves and also with selected

members of other AGs (Carling et al., 2002). In addition to the AG concept, several characteristics such as pathogenicity, host range, distribution in nature, physiological characteristics, and morphology have been used in order to distinguish the different species within the *R. solani* species complex (Kellens and Pneumans, 1991). The detection and identification of the different AGs are often determined by molecular biology (Bounou et al., 1999; Cubeta et al., 1996; Duncan et al., 1993; Gonzalez et al., 2006; Jabaji-Hare et al., 1990; Lees et al., 2002).

2.6.1. *Rhizoctonia solani* AG-3

To date, in the province of Québec, all *R. solani* isolates from infested potatoes (*Solanum tuberosum*) have been assigned only to AG-3 (Banville, 1989; Otrysko et al., 1985). On potatoes, *R. solani* causes stem canker that delays plant emergence and decreases the number and size of harvested tubers which can result in a reduction of total yield (Hide and Horrocks, 1994; Hide et al., 1996). There are two phases of the disease, stem canker and black scurf, jointly referred to as Rhizoctonia disease of potato. The former usually occurs early in the growing season in the form of lesions on the growing tips of sprouts, stems, or stolons leading to sprout and stem nipping. This can occur throughout the growing season. The second and most noticeable phase is the formation of the vegetative resting structures, the sclerotia, on tubers. These sclerotia are often referred to as black scurf. The accumulation of black sclerotia on tubers is initiated by the physiology of the plant. As the plant starts to die, the fungus begins forming sclerotia on tubers. The longer the potatoes remain in the soil after vine death, the more sclerotia will be formed on the tubers. The pathogen overwinters as sclerotia and mycelium on infected tubers, in plant residue, or in infested soils. High soil moisture, cool temperatures, high soil fertility, and a neutral to acid soil (pH of 7 or less) favour development of the disease.

Currently, a completely effective control strategies for this disease does not exist, but cultural and management practices that reduce the severity of the disease. No potato variety exists or has been developed with immunity for sprout

nipping and stem lesion phase. Some varieties show varying degrees of resistance to formation of sclerotia on tubers but none are completely resistant. Cultural and chemical controls have been used but they are not always efficient (Bains et al., 2002; Grosch et al., 2005). Crop rotation with cereals reduces both the incidence and severity of the disease. Of interest, *Rhizoctonia* does not compete exceptionally well with other microbes in the soil. Thus, increasing the rate of crop residue decomposition and the amount of organic matter in the soil decreases the growth rate of *Rhizoctonia*, while the utilization of chemical fungicides reduces the diversity of soil microorganisms and consequently the suppression of this pathogen (Garbeva et al., 2006).

2.6.2. Biocontrol of *R. solani*

For more than two decades, potential biological methods have been developed in order to reduce the impact of *R. solani* on crops and horticultural plants. These methods involve cultural practices and the use of biocontrol agents such as mycoparasites, bacteria, and non-pathogenic fungi.

Rhizoctonia solani does not compete exceptionally well with other soil organisms. Cultural methods such as crop rotation and solarization have been effective in reducing Rhizoctonia disease of potato. A three-year crop rotation with cereals is effective in increasing the diversity of soil organisms and decreasing *R. solani* inoculum threshold in the soil. The increase in soil temperature through solarization was also shown to be effective in reducing the development of *R. solani* (Bains et al., 2002; Garbeva et al., 2006; Secor and Gudmestad, 1999). Although the use of biocontrol agents showed great potential under controlled conditions but proved to be inconsistent as well as unreliable in the field mainly because the activity of these organisms varies according to the soil type, the environmental conditions, and the formulation (Brewer and Larkin, 2005).

Among the biocontrol agents that show promise are isolates of binucleate *Rhizoctonia* (BNR) species. They have been reported to be effective against Rhizoctonia disease in a broad range of plants including bean, potato, cucumber,

and cotton (Escande and Echandi, 1991; Gonzalez Garcia et al., 2006; Jabaji-Hare and Neate, 2005; Xue et al., 1998). Protection against Rhizoctonia disease by BNR isolates has been attributed to competition for nutrients at the infection sites (Escande and Echandi, 1991; Hwang and Benson, 2003), and/or eliciting systemic induced resistance (Jabaji-Hare and Neate, 2005; Wen et al., 2005; Xue et al., 1998). In addition, antagonists such as bacteria (Asaka and Shoda, 1996; Bagnasco et al., 1998; Hwang and Benson, 2002; Nielsen and Sorensen, 1997; Park et al., 1995; Quan et al., 2006; Sabaratnam and Traquair, 2002), yeast (El-Tarabily, 2004), and mycoparasites other than *Trichoderma* species (Benyagoub et al., 1994; El-Tarabily, 2004; Lewis et al., 1995a; Lewis et al., 1995b; Lewis and Larkin, 1997, 1998; Lewis et al., 1998; Lewis and Papavizas, 1992; Sun et al., 2006), alone or in combination with fungicide (Kondoh et al., 2001; Wang et al., 2005), have been shown to limit the development of *R. solani*.

Only a few studies have been reported on the potential efficacy of biocontrol agents, such as bacteria (Grosch et al., 2005) and fungi, such as binucleate *Rhizoctonia*, *T. harzianum*, *T. virens*, *Laetisaria arvalis*, *Verticillium biguttatum*, and *Penicillium* sp., (Beagle-Ristaino and Papavizas, 1985; Brewer and Larkin, 2005; Escande and Echandi, 1991; Lewis and Papavizas, 1992), in controlling the disease from *R. solani* AG-3, which is a pathogen for potato and tomato plants.

2.7. *STACHYBOTRYS ELEGANS*

This fungus belongs to the phylum Ascomycota and the class Ascomycetes. Within the genus *Stachybotrys*, there exists 15 species: *S. alternans, S. atra, S. bisbyi, S. chartarum, S. cylindrospora, S. dichroa, S. kampalensis, S. logispora, S. microspora, S. nephrospora, S. nilagirica, S. oenanthes, S. parvispora, S. sansevieriae,* and *S. theobromae* (Catalogue of life: 2006 Annual checklist; http:/annual.sp2000.org). *S. elegans* (Pidopl.) W. Gams (anamorph) is the accepted taxonomic name, but has several synonyms: *Hyalobotrys elegans* Pidopl., *Hyalostachybotrys bisbyi* Srinvi., *Hyalostachybotrys sacchari* Srinvi., *S.*

aurantia G.L. Barron, *S. bisbyi* (Srinvi.) G.L. Barron, and *S. sacchari* (Srinvi.) G.L. Barron (Catalogue of life: 2006 Annual checklist).

Of interest, the genus *Stachybotrys* is related to the order of *Hypocreales* to which the well-known mycoparasite *Trichoderma* spp. belongs (Castlebury et al., 2004). The most studied *Stachybotrys* species are *S. chatarum*, *S. alternans*, and *S. atra* which cause several illnesses in animals and humans including pulmonary hemosiderosis (bleeding in the lungs) in infants and immune competency dysfunction (Li et al., 2002). The toxicity of *S. chartarum* is directly related to the production of several secondary metabolites, including the mycotoxins trichothecenes, trichodermol, and atranones (Andersen et al., 2003; Jarvis, 2003). Trichothecenes are also produced by few *Trichoderma* species and their implication in mycoparasitism has been reported (Di Pietro et al., 1993; Lumsden et al., 1992; Nielsen et al., 2005; Papavizas, 1985; Wilhite and Straney, 1996). The trichothecenes are potent inhibitors of protein and DNA synthesis (Cundliffe and Davies, 1977; Ueno et al., 1973; Ven Murthy et al., 1985).

S. elegans is a necrotrophic mycoparasite of the soilborne plant pathogen *R. solani* killing hyphae as well as sclerotia (Benyagoub et al., 1994). Contrary to *S. chartarum,* the species *elegans* does not produce macrocyclic trichothecenes, trichodermol, or atranones (Andersen et al., 2002). The process of mycoparasitism of *R. solani* cells by *S. elegans in vitro* is well characterized by a) contact and appressed growth, b) coiling, c) penetration of *R. solani* hyphae and internal growth, and d) subsequent sporulation outside the host (Benyagoub et al., 1994). Wall thickness and melanized cell walls do not stop the progression of *S. elegans*. Hyphal growth into the cytoplasm of *R. solani* leads to the granulation of the cytoplasm and unavoidable death of the *R. solani* host cells (Benyagoub et al., 1996).

Stachybotrys elegans is a rich source of glucanolytic, chitinolytic, proteolytic and cellulolytic enzymes. The availability of these proteins and their encoding genes allows us to determine how best to use them in pest management strategies. Our laboratory has shown that the mycoparasite *S. elegans* is capable of degrading *R. solani* cell walls and releases large amounts of several β-1,3-

glucanases, and to a lesser extent chitinases, into culture medium previously amended with *R. solani* cell walls as a carbon source (Tweddell et al., 1994). However, the production of β-1,3-glucanases is much higher than chitinases. This is not surprising since cell wall of *R. solani* contains significantly more β-1,3-glucan polymers than chitin. At least four β-1,3-glucanases with molecular masses of 180, 110, 94 and 75-kDa (Archambault et al., 1998a), two exochitinases (one identified as acetylhexasaminidase), and one endochitinase are produced when *S. elegans* is grown on purified chitin or cell walls of *R. solani* (Taylor et al., 2002). Recent purification of the 94- and 75-kDa β-1,3-endoglucanases and the exochitinase 68-kDa β-1,4-acetylhexasaminidase referred to as NAG-68, enabled us to confirm that the enzymes are secreted during mycoparasitism (Archambault et al., 1998a; Taylor et al., 2002; Tweddell et al., 1995) and are antifungal. The 94-kDa glucanase has been shown to be activated by a protease (Archambault et al., 1998b). The recently purified exochitinase NAG-68 is induced when *S. elegans* was grown on purified cell wall fragments of *R. solani* as well as during the mycoparasitic interaction when both host and mycoparasite were grown on synthetic medium supplemented with or without a supplemental carbon source (Taylor et al., 2002). These results suggest that NAG-68 is involved in the mycoparasitism of *R. solani* by *S. elegans*.

Only one cloned gene, *sechi44*, has been characterized from *S. elegans* (Morissette et al., 2003). The gene sequence and its expression are highly similar to the endochitinase-encoding *ech42* from *T. atroviride*. *sechi44* is involved in mycoparasitism and linear growth of *S. elegans*. The genetic regulation of *sechi44* during the mycoparasitic process is not well understood, even with the substantial knowledge acquired from research on *Trichoderma* species. It is imperative to identify other genes involved in this process in order to improve our understanding of the mycopasrasitic process and the use of mycoparasites.

2.8. GENE EXPRESSION STUDY

In order to better understand the mycoparasitic process, more information on the genes directly or indirectly involved in the the mycoparasitic process might

allow the development of improved biocontrol agents, new protein formulations, or resistant transgenic crops. Gene expression studies can be accomplished by different techniques related to PCR.

2.9. POLYMERASE CHAIN REACTION

PCR was developed by Kary Mullis in the mid 1980s. This revolutionary technique allows the enzymatic amplification of nucleic acids in large copies and the qualitative studies of the genome and the transcriptome (RT)-PCR) of any organisms. In 1992, Higuchi et al. developed real-time PCR in which the amplification is monitored by the addition of fluorescent dye in the PCR reaction. Assuming that the amount of product added in the reaction is proportional to the amount of product at the end of the amplification, it is possible to quantify the amount of the product at the beginning of the reaction. In 1996, Gibson et al. developed real-time RT-PCR in order to monitor the gene expression from a small sample of RNA. This had opened many avenues in which several PCR-related techniques have been developed such as rapid amplification of cDNA ends (RACE)-PCR, suppression subtractive hybridization (SSH), and microarray. PCR and real-time PCR techniques have been extensively used to study plant pathology, plant-microbe interactions and, to a lesser extend, microbe-microbe interactions.

2.9.1. Rapid amplification of cDNA ends (RACE)-PCR

RACE-PCR allows the amplification and cloning of both ends (5' and 3' ends) of a cDNA, giving rapidly the full-length cDNA from a transcript. This method was first developed by Frohman et al. (1988) and it was improved with time by several users. The major modification was the ligation of adaptors at the both ends of the full cDNA (Chenchik et al., 1996). The adaptor sequences anneal with their specific primers used in combination with gene specific primers. This technique was successfully used in this thesis (Chapter 3) to clone the full-length cDNA of an endochitinase gene *sechi44*, and in chapter 6 to clone the full-length

cDNAs of four uncharacterized novel genes *seglu*, *selec*, *sepol*, and the 3'end of the *se151* gene.

2.9.2. Quantitative real-time reverse transcription (QRT)-PCR

The application of QRT-PCR in plant pathology and in plant-microbe interaction studies increased substantially since the beginning of 2000. Compared to Northern blot analysis, this technique allows the accurate quantification of gene expression, especially when RNA amounts are limited. Although the use of QRT-PCR is not straightforward. Two main factors that can affect the quantification of gene expression are the choice of the gene for normalization and the method of calculation for gene expression.

2.9.2.1. Choice of the gene for normalization

The optimal gene to be used for normalization purposes should (i) have a constant expression irrespective of the treatments, (ii) be verified for stability and constant expression prior to its selection as the housekeeping gene (HKG), and (iii) must reflect a high specificity towards the target organism. This is of utmost importance especially when gene expression studies involve more than one organism belonging to the same taxonomic group. In this study, the fungal species whose gene expression was studied belongs to a different taxonomic classes. Howevr,their genomes are not completely sequenced, except for few genes encoding the ribosomal sequences, such as large subunit or small subunits, and mitochondrial sequences, making the selection of HKGs limited to the few available sequenced genes.

2.9.2.2. Gene expression quantification

In QRT-PCR, gene expression can be measured in terms of either absolute or relative quantification. Absolute quantification is presented as number of copies of a transcript based on standard curves. Relative expression is presented as the relative change in the expression of a gene compared to HKG, and standard curves are not necessary. The relative quantification method is easier to perform

than absolute quantification and allows comparison of expression data from different QRT-PCR experiments (Pfaffl, 2004). This method was applied for gene expression analyses throughout this study.

Various methods have been developed for the relative quantification of gene expression including: 1) standard curve method which requires the calculation of the number of transcript copies relative to the calibrator designated as 1-fold (Livak, 1997); 2) comparative C_T method, $2^{-\Delta\Delta Cp}$ method, which is based on the difference of the crossing point (C_p) of the experimental and control sample (Livak, 1997). This method assumes that all amplifications have the same efficiency; 3) the Pfaffl model, is well-known and extensively used method. It is based on the PCR efficiency and the C_P deviation of the investigated transcripts (Pfaffl, 2001). In addition to the calibrator, this method includes the normalization of the expression by a HKG. In 2002, Pfaffl et al. developed the program REST which calculates the ratio based on E and mean ΔCP of sample vs. control group, and tests the significance by a pair-wise fixed reallocation randomisation test; 4) Q-Gene is a software in which the raw data of QRT-PCR are computed to give relative expression after normalization with HKG and calibration with control (Muller et al., 2002); finally, 5) the method of Liu and Saint (2002) is very similar to $2^{-\Delta\Delta Cp}$ method (Livak, 1997) with correction for PCR efficiency. This efficiency is calculated from the slope of each amplification curve so that production of standard curves is not necessary.

The standard curve and the comparative C_T methods assume that PCR efficiency is near to perfect, which is rarely the case. REST and Q-Gene software, which are presented as a macro Excel sheet, has been developed for medical research in which only one kind of cell or organism is present in samples and is not suitable when RNA represents more than one organism.

2.9.3. Identification of diffrentially expressed genes

Genes involved in mycoparasitism should be differentially expressed when the mycoparasite and the host grow in the vicinity of each other as compared to when they do not. Different methods can be used to analyse differential gene

expression. These include differential display, cDNA macroarrays, cDNA microarrays, serial analysis of gene expression (SAGE and SuperSAGE), SSH, and differential screening (Diatchenko et al., 1996; Gold et al., 2001; Kuhn, 2001; Zhang et al., 1998). All these methods apply PCR technologies.

2.9.3.1. Suppression subtractive hybridization (SSH)

SSH was developed by Diatchenko et al. (1996) from another technique called suppression PCR. This method consists of comparing two populations of mRNA and obtaining clones of genes that are expressed in one population but not in the other. Both mRNA populations are converted into cDNA. We refer to the cDNA that contains specific (differentially expressed) transcripts as "tester", and the reference cDNA as "driver". Tester and driver cDNAs are hybridized, and the hybridized sequences are discarded. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent in the driver. The differentially expressed RNAs can be identified by sequence and hybridization analysis (library screening).

This technique has been improved by incorporating a "suppression" PCR and a single round of subtraction (Diatchenko et al., 1996). Because of this suppression step, the technique is now referred as suppression subtractive hybridization (SSH), and achieves up to 1000-fold enrichment of differentially expressed cDNAs, including low-abundance species.

SSH has been used to study fungal gene expression in plant-fungus interaction including the pathosystems *Zea mays/Colletrotrichum graminicole* (Sugui and Deising, 2002), *Triticum aestivum/Puccinia graminis* (Broeker et al., 2006), and *T. aestivum/Fusarium graminearum* (Bernardo et al., 2007; Goswami et al., 2006), to study differential expression between wild-type and mutant strains of *Hypocrea jecorina* that are defective in induction of cellulose gene expression (Schmoll et al., 2004), and to demonstrate gene expression when fungi are grown under different growth conditions or culture media as in the case of *Metarhizium anisopliae* on nitrogen-limiting media and rich media (Small and Bidochka, 2005), *Aspergillus ochraceus* growing on ochratoxin A inducing and non-

inducing media (O'Callaghan et al., 2003), and *Penicillium chrysogenum* on repressing and non-repressing carbon source (Castillo et al., 2006). Only one study was conducted on a fungus-fungus interaction system, *Trichoderma hamatum* and *Sclerotinia sclerotiorum* in which SSH has been successfully applied (Carpenter et al., 2005).

2.9.3.2. Differential screening of a subtracted library by microarrays

In principal, differential screening (DS) can be used to identify any differentially regulated gene. In practice, this method works well only when the mRNA of interest comprises more than 0.05% of the total mRNA in one cell and less than 0.01% in the other (Sambrook et al., 1989). SSH improved greatly the identification of rare mRNAs (Kuhn, 2001). Considering the advantages of applying DS, it is surprising that its use in fungal gene analysis is imited to a few studies (Birch et al., 1999; O'Callaghan et al., 2003; Schmoll et al., 2004; Sugui and Deising, 2002). The microarray analysis method has been developed recently by van den Berg et al. (2004). The equations are based on comparing the intensity of the spots of three cDNA libraries obtained from the SSH, the subtracted tester (ST), the unsubtracted driver (UD), and the unsubtracted tester (UT), and calculating an expression ratio.

$$UT/UD = \text{antilog}\,(ER1 - ER2) \text{ in base } 2 \qquad \text{Equation [2.1]}$$

Where:

$$ER1 = \tfrac{1}{2}\,[(\log_2 \text{Cy3 ST/Cy5 UD}) - (\log_2 \text{Cy3 UD/Cy5 ST})]$$

$$ER2 = \tfrac{1}{2}\,[(\log_2 \text{Cy3 ST/Cy5 UT}) - (\log_2 \text{Cy3 UT/Cy5 ST})]$$

ER1 and ER2 are enrichment ratios of ST/UD and ST/UT, respectively, compiled from slides hybridized with ST and UD, and ST and UT, respectively. An UT/UD >1 is considered up-regulated.

CONNECTING STATEMENT BETWEEN CHAPTERS 2 AND 3

Chapter 3 reports on the isolation of an endochitinase-encoding gene, *sechi44*, from the mycoparasite *Stachybotrys elegans*. PCR primers were designed based on chitinase-encoding genes from *Trichoderma* spp. Specific primers were designed on this amplified amplicon. Rapid amplification of cDNA ends (RACE)-PCR was then performed in order to clone the entire sequence of the cDNA. The number of copies present in the *S. elegans* genome was determined by southern blots. *sechi44* expression during the interaction of *S. elegans* and *Rhizoctonia solani* was quantified by quantitative real-time reverse transcription (QRT)-PCR. The expression of *sechi44* was normalized using the β-tubulin-encoding gene from *S. elegans* as the HKG. Since no standard curves were developed, the method of Liu and Saint (2002) was used to calculate the efficiency of amplification. The results of this section are published in Fungal Genetics and Biology (2003, Vol. 39, pp. 276-285). I designed the experimental set-up, conducted all the experiments, and wrote the manuscript. The contributions of the co-authors were as follows: Professor S. Jabaji-Hare provided supervision, and funding throughout this study. She also made suggestions and corrected the manuscript. Professor B. T. Driscoll conducted the phylogenetic analysis, made suggestions, and revised the manuscript.

CHAPTER 3

Molecular cloning, characterization and expression of a cDNA encoding an endochitinase gene from the mycoparasite *Stachybotrys elegans*.

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3.1. ABSTRACT

Stachybotrys elegans is a mycoparasite of the soilborne plant pathogenic fungus *Rhizoctonia solani*. The mycoparasitic activity of *S. elegans* is correlated with the production of cell wall-degrading enzymes such as chitinases. This report details the cloning by RACE-PCR, and characterization of a full-length cDNA clone, *sechi44*, that appears to encode an extracellular endochitinase. An analysis of the *sechi44* sequence indicates that this gene contains a 1269 bp ORF and encodes a 423 aa polypeptide. The SECHI44 protein has a calculated molecular weight of 44.1 kDa and pI of 5.53. Since the SECHI44 protein also appears to encode a signal peptide, an extracellular location for the corresponding protein is predicted. Comparison of SECHI44 sequence with known sequences of fungal endochitinases revealed that SECHI44 is grouped with endochitinases from other mycoparasites. Real-time quantitative RT-PCR analysis, showed an elevated level of expression of *sechi44* (21 fold) in chitin-rich (induced) as compared to no-carbon (non-induced) culture conditions. In dual culture, the temporal expression of *sechi44* increased after 2 days of contact with *R. solani*, reaching a 10-fold increase after 9 days, followed by a decrease to basic expression level at 12 days. Interestingly, inhibition of *sechi*44 expression was observed when *S. elegans* hyphae were in close proximity with *R. solani* hyphae.

Keywords: *Stachybotrys elegans*, *Rhizoctonia solani*, endochitinase, *sechi44*, mycoparasite, hydrolytic enzyme, fungi, RACE-PCR, gene cloning, real-time quantitative RT-PCR.

3.2. INTRODUCTION

Despite numerous publications on the utilization of antagonistic (parasitic) microorganisms for biocontrol, the mechanisms by which they control plant pathogenic fungi are not well understood. Among the proposed mechanisms of action for mycoparasites is the production of cell wall-degrading enzymes (CWDEs), such as chitinolytic enzymes, β-1,3 glucanases and proteases, that are able to lyse fungal cell walls (Lorito, 1998). Such enzymes are most frequently considered in biocontrol (Elad and Kapat, 1999; Lorito et al., 1996a). Most of the relevant studies on biocontrol of fungi by mycoparasites, and the role of CWDEs in this process has been done using a few strains belonging to the genus *Trichoderma* (El-Katatny et al., 2001; Rey et al., 2001). Purified CWDEs are effective fungicides against many important plant pathogens, likely via their ability to lyse various fungal structures such as hyphae, conidia, chlamydospores, and sclerotia (Lorito et al., 1996a; Tsahouridou and Thanassoulopoulos, 2001). Antifungal activity is synergistically enhanced when different CWDEs are used together (Lorito et al., 1996c; Schirmbock et al., 1994) or when combined with plant pathogenesis-related proteins (Lorito et al., 1998). As mycoparasites specifically attack other fungi but not plants, they represent a promising source of novel antifungal genes.

While many plant and microbial chitinase genes have been described, only a few have been reported from mycoparasites. The majority of known mycoparasite chitinase genes are from *T. harzianum*, *T. atroviride* and *T. virens*. Ten chitinase-encoding genes have been cloned and characterized from *Trichoderma* spp., namely *chit 33* (Kim et al., 2002; Limon et al., 1995), *ech42* (Carsolio et al., 1994; Hayes et al., 1994; Kim et al., 2002), *chit42* (Garcia et al., 1994), *exc1* and *exc2* (Draborg et al., 1995), *chit36* (Viterbo et al., 2001) and *nag1* (Kim et al., 2002; Peterbauer et al., 1996). The endochitinase encoding gene *ech42* and the protease encoding gene *prb1*, coding for Ech42 and basic protein Prb1 respectively, were highly expressed when *Trichoderma* strains are grown on chitin-rich medium or in dual cultures with a host (Carsolio et al., 1994; Geremia et al., 1993). Furthermore, transgenic plants containing *ech42* and transformed

Trichoderma strains carrying multiple copies of *prb1* showed a higher resistance to several fungi (Flores et al., 1997; Lorito et al., 1998). These results suggest that these genes are directly involved in mycoparasitism. Despite these advances, we still have not observed the total diversity of genes encoding CWDEs produced by other fungal mycoparasites.

The mycoparasite *Stachybotrys elegans* (Pidopl.) W. Gams is a rich source of glucanolytic, chitinolytic, proteolytic and cellulolytic enzymes. To date, we have characterized two β-1,3-glucanases (Archambault et al., 1998b; Tweddell et al., 1995), one β-*N*-acetylhexosaminidase (Taylor et al., 2002), and one β-1,4-glucanase (Tweddell et al., 1996). As with *Trichoderma* species, *S. elegans* is capable of degrading *Rhizoctonia solani* cell walls (Benyagoub et al., 1994) and releases the CWDEs, β-1,3-glucanases and chitinases, into culture medium amended with *R. solani* cell wall fragments, or with chitin as a carbon source (Tweddell et al., 1994). There have been no previous reports on the characterization of a *S. elegans* gene encoding a CWDE.

In this study, we report on the cloning, molecular characterization and expression of an endochitinase gene from *S. elegans*. The *sechi44* gene was cloned via the rapid amplification of cDNA ends (RACE)-PCR technique. Expression studies, using real-time quantitative RT-PCR, indicated that *sechi44* levels are differentially expressed when *S. elegans* was grown under induced and non-induced conditions and during its mycoparasitic interaction with *R. solani*.

3.3. MATERIALS AND METHODS

3.3.1. Organisms and culture conditions

Starter cultures of the ascomycete *Stachybotrys elegans* (ATCC 188825), *Trichoderma harzianum* strain CECT 2413 (CBS 354.33) and the basidiomycete *Rhizoctonia solani* AG-3 (ATCC 10183) were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan) at 24°C (room temperature) for 5 days. Homogenized mycelia from starter cultures of *S. elegans* and *T. harzianum* were used to obtain sufficient mycelial growth on liquid media for DNA and RNA extractions. Inoculation of potato dextrose broth (PDB; Difco Laboratories) and

minimal synthetic medium containing no carbon source (MSM, Tweddell *et al.*, 1995) was done by adding 2 ml of homogenized mycelia of *S. elegans* and *T. harzianum* to 250 ml Erlenmeyer flasks containing 50 ml of PDB, or MSM. The MSM composition (g/L): 0.2 g $MgSO_4.7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCL, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.002 g $MnSO_4$, 0.002 g $ZnSO_4$, and 0.01 g biotin. The pH was adjusted to 5.2 with HCl. For induction of chitinases (induced conditions), purified colloidal chitin from crab shells (1.0 g/L, Sigma Chemical Co., St. Louis, Mo.) was added to MSM as sole carbon source (Tweddell et al., 1995). The flasks were incubated with shaking (110 rpm) at 24°C for 7 days for PDB medium and at 130 rpm for 3 days at 28°C for MSM media. Culture filtrate was collected by filtration through Whatman no. 1 filter paper, flash-frozen in liquid nitrogen, lyophilized (for DNA extraction only) and stored at –80 °C.

3.3.2. Isolation of DNA and RNA

Total genomic DNA was isolated from freeze-dried mycelia of *S. elegans* and *T. harzianum*, according to the standard method of (Lee and Taylor, 1990) and (Leclerc-Potvin et al., 1999) with the following modifications: the DNA was subjected to two rounds of phenol-chloroform/isoamyl alcohol, and the isolated DNA was treated with 20 µg RNase-ribonuclease A (Sigma) for 90 min at 37°C.

For RNA extraction, frozen mycelia of *S. elegans* and *R. solani* were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA from 100 mg of powder was isolated using the RNeasy Plant Mini Kit (QIAGEN, Mississauga, ON) and treated with RNase-free DNase I (QIAGEN) according to the manufacturer's recommendations. Contamination of RNA samples with DNA was verified by PCR amplification on total RNA with SechiF and SechiR primer set (Table 3.1). Prior to RACE-PCR and retrotranscription experiments, the concentration and purity of RNA were checked by their absorbance at 260 and 280 nm.

3.3.3. Confrontation assays

For *in vitro* expression of the endochitinase gene *sechi44* during mycoparasitism of *R. solani* by *S. elegans*, a 1 cm^2 agar piece of *S. elegans* starter culture was placed on a plate of MSM supplemented with 1% agar (MSMA; Gellan Gum, Kelco, San Diego, California) and covered with permeable cellophane (PUT79; Flexel Inc., Atlanta, GA), and then a 1 cm^2 agar piece of *R. solani* starter culture was placed on the same plate at a distance of 6 cm from *S. elegans*. This set-up allowed the two fungi to grow towards, and eventually contact each other. Total RNA (from duplicate plates) was extracted from (i) hyphae of both fungi when they were distanced 1.0 and 0.5 cm apart prior to contact, (ii) a 4-cm strip of both fungi at the zone of interaction at 2, 3, 9 and 12 days after their hyphae intermingled, (iii) hyphae of 7 day-old pure cultures of each of *S. elegans* and *R. solani* from MSMA (control), and (iv) hyphae of 7 day-old pure cultures of *S. elegans* growing on chitin (MSM amended with chitin, induced conditions). Total RNA was treated with RNase-free DNase I (QIAGEN), and 500 ng of total RNA was retrotranscribed into cDNA using the Omniscript Reverse Transcriptase kit (QIAGEN) according to the manufacturer's recommendations. The experiment was repeated twice.

3.3.4. Primers and primer design

To obtain the entire cDNA for the chitinase gene, several primer sets were designed for different stages of this study (Table 3.1). All primers were tested for self-complementarity, complementarity between both primers, and theoretical melting temperature was determined using the software DNAman v. 4.13. Primer pair chiaF and chiaR were designed from the alignment of highly conserved sequence regions of endochitinase genes of *T. harzianum* T25-1 (acc. no. U49455), *T. harzianum* CECT 2413 (acc. no. S78423), *T. atroviride* IMI206040 (acc. no. X79381) and *Aphanocladium album* (acc. no. X64104). chiaF/chiaR were used in PCR reactions to amplify putative products from genomic DNA with an expected size of 913 bp (Table 3.1). For RACE-PCR and Reverse-Transcriptase (RT)-PCR reactions, gene specific primers (GSP) were designed

based on the sequence of the putative product from *S. elegans* genomic DNA that was obtained with chiaF/chiaR. The GSP primers were SechiF for the 3' RACE, and SechiR and SechiRnested for 5' RACE (Table 3.1). For sequencing reaction, the primers InDanF and InGreR were designed in order to fill the gap at the 3'end of the cDNA sequence (Table 3.1).

The primer ITS1-F, which is specific for higher fungi (Gardes and Bruns, 1993), and the universal primer ITS4 (White et al., 1990), were used together as a positive control as they are expected to amplify the ITS region of the 5.8S ribosomal DNA from Basidiomycetes and Ascomycetes.

3.3.5. Polymerase Chain Reaction (PCR) amplification

The PCR reactions for the universal primers (ITS1-F/ITS4) and chiaR/chiaF primers were performed in a 25-µl PCR reaction mixture [0.20 mmol of each dNTP, 1.5 mmol of $MgCl_2$, 0.2 µmol of each primer, 1x of PCR buffer (Gibco-BRL, Burlington, ON), 1U of Taq DNA polymerase (Gibco-BRL)] and 5 ng of DNA of the appropriate organism. Samples were overlaid with mineral oil and amplified in a Perkin Elmer DNA thermal Cycler model 480. For ITS1-F/ITS4, the PCR conditions consisted of an initial denaturation at 93°C for 3 min, 35 cycles of amplification consisted of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Then further extension at 72°C was performed for 10 min. The PCR reaction using chiaR/chiaF consisted of the same conditions as those used for the universal primers for *S. elegans* but an annealing temperature of 62°C for *T. harzianum* DNA was used. The amplified PCR products (913 bp and 896 bp) of interest from both fungi were cleaned with PCR Purification kit (QIAGEN) for *T. harzianum* and with QIAquick gel extraction kit (QIAGEN) for *S. elegans*, cloned using Zero Blunt PCR Cloning Kit (Invitrogen, Burlington, ON) following the manufacturer's recommendations, and sequenced (ABI PRISM, model 310).

3.3.6. Rapid amplification of cDNA ends (RACE)-PCR

Total RNA (1.0 µg) from 7-day-old induced cultures of *S. elegans* was used to synthesize anchored double stranded cDNA templates, and further used to amplify the 5' and 3' ends of cDNA clone as described by the GENE RACER kit (Invitrogen), using the designed GSP primer set (Table 3.1). Amplification was performed in 25-µl reaction mixture [0.20 mmol of each dNTP, 1.0 mmol of $MgSO_4$, 0.2 µmol of each primers, 1x of PCR buffer (Gibco-BRL)], 2.5U of Taq DNA polymerase (Gibco-BRL) and 1 µl of template. Samples were overlaid with mineral oil. The PCR reaction consisted of an initial denaturation at 94°C for 2 min, 5 cycles of 30 sec at 94°C and 2 min at 72°C, 5 cycles of 30 s at 94°C and 2 min at 70°C, 25 cycles of 30 s at 94°C, 30 s at 68°C and 2 min at 72°C, and an extension cycle at 72°C for 10 min. Nested RACE-PCR was performed for the 5' end of the cDNA only with an initial denaturation at 94°C for 2 min, 25 cycles of 30 s at 94°C, 30 s at 65°C and 2 min at 68°C, and an extension cycle at 68°C for 10 min. Amplification was performed in 25-µl reaction mixture, the same as for RACE-PCR, except that the 5'-end RACE products (diluted $1x10^{-4}$) were used as template. The RACE-PCR cDNA amplified products of interest were sub-cloned in plasmid pCR®-TOPO following TOPO Cloning Kit (Invitrogen) recommendations.

3.3.7. Sequencing and analysis of the chitinase gene

The sequence of *T. harzianum* product obtained with chiaF/chiaR primer pair was submitted to NCBI programs for homology with *T. harzianum ech42* gene (Blastn), while the sequence of *S. elegans* product was submitted to NCBI programs for homology with other chitinase genes (Blastn) and proteins (Blastx). This sequence was used to design GSP for RACE-PCR as described above.

For sequence analysis of the chitinase cDNA gene, both cloned PCR products (5'end and 3'end) were sequenced (ABI PRISM, model 310 and ABI PRISM 3100; Nucleic Acid Analysis and Synthesis Laboratory, Laval University, Quebec, Canada). cDNA sequence analysis was carried out using the following

programs available at various sites on the World Wide Web: multiple sequence alignments using Clustal W on the Biology Work Bench server (http://biowb.sdsc.edu/CGI/BW.cgi#!) and DIALIGN on the ExPASy server (http://www.expasy.ch/); protein translation of the cDNA sequence, and nucleotide and protein sequence analysis were done using the ExPaSy programs Translate, Scan Prosite, PSORT, Signal P and Compute pI/Mw (http://www.expasy.ch/); Blastx and Blastn database searches using the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). Restriction analysis was done using the software DNAman v. 4.13. Phylogenetic analysis of SECHI44 and other related endochitinase amino acid sequences was done using MacVector v. 7.0 (Oxford Molecular Ltd., Genetics Computer Group, Madison WI), and selected algorithms therein. Phylogenetic trees were reconstructed by the neighbor-joining method with systematic (tie) breaking (Saitou and Nei, 1987). *N*-acetylhexosaminadase (CHIT73) of *T. atroviride* (AAB50829) was used as an outgroup. Distances were calculated using the uncorrected ("p") method, and gap sites were ignored. Phylogenetic trees were subjected to bootstrap analysis with 1000 replications (Felsenstein, 1985).

3.3.8. Chitinase cDNA amplification by reverse transcription (RT)-PCR

RT-PCR reactions were performed on cDNA templates prepared from total RNA that was extracted from confrontation assays, non-induced, and induced cultures of *S. elegans* and *R. solani* using the GSP primer pair, SechiF and SechiR (Table 3.1). Amplification was performed in 25-µl reaction mixture containing the same final concentration as for regular PCR reaction. The same PCR program used for reaction with universal primers was used for RT-PCR.

3.3.9. Expression analysis by real-time quantitative (Q)RT-PCR

sechi44 transcripts were quantified by real-time QRT-PCR using LightCycler (Roche) and SYBR Green master mix as per manufacturer's specifications (QIAGEN). Quantification was based on a 592 bp amplicon generated using the GSP primer pair. Reactions were set up in microcapillary

tubes using the following final concentrations: 0.4 μmol each of SechiF and SechiR primers (Table 3.1), 1x SYBR Green master mix, and 2 μl of cDNA (one fiftieth of total cDNA). Amplification conditions were 95°C for 15 min (hot start), followed by 70 cycles of 94°C for 15 s, 68°C for 20 s, and 72°C for 25 s. Following amplification, a melting curve program (65-95°C with a heating rate of 0.1°C/s) and a cooling step to 40°C were added. RT-PCR reactions for each sample were replicated three times, means and standard deviations were calculated, and data were analyzed using LightCycler analysis software. The cycle threshold (C_T) was measured at the fixed threshold of 0.2. The number of cDNA transcripts was normalized against the expression of the housekeeping ascomycete β-tubulin gene (Glass and Donaldson, 1995) that was measured in parallel PCR runs using the primers Bt2a and Bt2b (Table 3.1). Real-time RT-PCR data are presented as relative expression using the equation $R_{N,b}/R_{N,a} = (1+E)^{-\Delta\Delta C}{}_T$ where R_n is the reporter fluorescence at cycle n, $R_{N,b}/R_{N,a}$ is the normalized R_0 for sample a (i.e., the gene of interest, *sechi44*) and b (i.e., the reference gene, β-tubulin), and $\Delta\Delta C_T$ = (C_T *sechi44* – C_T β-tubulin in experimental samples) – (C_T *sechi44* – C_T β-tubulin in a control sample) (Liu and Saint, 2002). In this experiment, the control sample is the *sechi44* cDNA transcripts expressed when *S. elegans* is grown under no carbon source culture conditions (MSM).

3.3.10. Genomic southern blot analysis

Total genomic DNA (20 μg) from *S. elegans* was digested to completion with the following restriction enzymes: *Bam*HI, *Dra*I, *Kpn*I, and *Sma*I, electrophoresed on 0.8% agarose gel and blotted onto Hybond-N membrane (Amersham, Baie d'Urfé, QB) using capillary transfer. The probe (*psech*) consisted of the partial chitinase gene fragment that was amplified using the GSP primers and *S. elegans* DNA. Labelling of the probe was performed using the T7 QuickPrime (Amersham) according to the manufacturer's recommendations. Hybridization was performed at 52°C in a preheated phosphate buffer recommended by the membrane manufacturer for 20 h. The membrane was

washed following the membrane manufacturer's recommendations, and exposed to K-screen (Kodak) for 16 h.

3.3.11. Nucleotide sequence accession number

The cDNA and gDNA nucleotide sequences data for *sechi44* have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession no. AF516397 and AY218835, respectively (Appendix I).

3.4. RESULTS

3.4.1. Isolation of cDNAs encoding *sechi44* from *S. elegans*.

On the basis of the alignment of the conserved regions of endochitinase gene sequence information, the primer pair chiaF/chiaR amplified a product of 913 bp in *T. harzianum* (positive control) and 896 pb in *S. elegans* (data not shown). In the case of *S. elegans*, four other less intense products were detected. Sequencing of the 913 bp product from *T. harzianum* showed that it shares 98% similarity with the endochitinase gene *ech42*. Blastn search of 896 bp from *S. elegans* indicated that the sequence had significant similarity with several endochitinase genes of *Trichoderma* spp. (80-83%), *Metharhizium anisopliae*, *Aphanocladium album*, and *Coniorytium minitans*. Blastx search indicated that the sequence had an 87% similarity with several *Trichoderma* spp. (70% identity), and with other fungi including the mycoparasites *Metharhizium* spp., *A. album,* and *C. minitans.*

The gene specific primers, SechiF, SechiR and SechiRnested (Table 3.1) were used to perform RACE-PCR with cDNA template from chitin-induced cultures of *S. elegans* (Fig. 3.1). The amplified product from the 5'end of the cDNA template was 652 bp in length (Fig. 3.1, lane 2), and the 3'end product was 1365 bp in length (Fig. 3.1, lane 3). Both products were sequenced and aligned to yield the complete *sechi44* cDNA of 1751 bp (Fig. 3.2) that contained a 1269 bp open reading frame (ORF) initiating with an ATG codon at nucleotide position 121 and terminating with a TGA stop codon at nucleotide position 1390. The deduced protein (SeCHI44) was composed of 423 amino acids (Fig. 3.2), and had

a calculated molecular weight of 46 kDa. The *sechi44* cDNA encodes an apparent signal peptide sequence of 20 amino acids with a typical signal cleavage site between Gly-20 and Phe-21 in the endochitinase SeCHI44 preprotein (Fig. 3.2). Thus, the mature excreted protein has a predicted molecular weight of 44.1 kDa and a pI of 5.53. The deduced SeCHI44 protein contains 2 protein kinase C phosphorylation sites, 6 casein kinase II phosphorylation sites, 9 N-myristoylation sites, 1 amidation site and 1 chitinase family 18 active site (Fig. 3.2). Southern analysis performed at high stringency indicated that the gene is present as a single copy in *S. elegans* genome (data not shown).

3.4.2. Sequence analysis

The deduced amino acid sequence of the *sechi44* gene product (SeCHI44) has 71% identity/84% similarity with *T. harzianum* Ech42 and *A. album* CHI1 endochitinases, and 70% identity/83% similarity *T. harzianum* CHIT42 and CHI1 endochitinases (Fig. 3.3). SeCHI44 also shares significant similarity with endochitinases from *Trichoderma* spp. and another mycoparasite, *C. minitans.* The phylogenetic tree shows that all nodes have high (>80%) bootstrap support (Fig. 3.4). The two *T. harzianum* chitinases are very similar and cluster together with the *T. atroviride* endochitinase. Neither the SeCHI44 nor the *A. album* chitinase cluster with any of the other known fungal chitinases, and SeCHI44 appears to be the most deeply-branched protein within the chitinases.

The partial genomic DNA (gDNA) sequence of 896 bp from *S. elegans* corresponded to the cDNA sequence from the nucleotide position 299 to 1078. gDNA sequence analysis identified two introns (i1 and i2). Intron 1 (i1) is 57 bp in length starting at nucleotide position 58 and ending at 114 of the gDNA, which is located between nucleotide position 357 and 358 of the cDNA sequence. The second intron (i2) is 62bp in length, and starts at nucleotide position 165 and ends at 226 of the gDNA sequence. This intron is located between nucleotide position 406 and 407 of the cDNA sequence. Because of the presence of i2, cDNA and gDNA amplification with SechiF and SechiR primers yielded two products of different lengths; 592 bp and 654 bp, respectively (data not shown). This enabled

us to distinguish the cDNA product from that amplified from gDNA during expression experiments.

3.4.3. Expression analysis

To test the expression of *sechi44*, RT-PCR was performed using cDNA prepared from RNA of *S. elegans* grown under induced and non-induced conditions and during dual interaction with *R. solani*. Different levels of the 592 bp amplification product were detected under non-induced conditions, induced conditions, and at different times during confrontation assays (Fig. 3.5). Differential expression of *sechi44* was detected when *S. elegans* was grown on non-induced (Fig. 3.5A, lane 3) and induced conditions (chitin-rich medium and dual culture) (Fig. 3.5A, lanes 4 and 7-9). *sechi44* expression was not detected in *R. solani* (Fig. 3.5A, lane 2). Quantification of the transcription levels of *sechi44* using real-time RT-PCR (Fig. 3.5B) showed that the levels were ~ 21 fold higher under chitin-rich (induced) conditions as compared to no carbon conditions (MSM, non-induced). During confrontation assays, there was a temporal progressive increase in gene expression from basal levels after 2 days of contact to a ~10 fold increase after 9 days of prolonged contact. The levels of expression were below the basal levels of expression after 12 days of contact, and when *S. elegans* was 1.0 or 0.5 cm away from *R. solani* (Fig. 3.5B).

Table 3.1. Primers used in different PCR and sequencing reactions.

Name	Primers (5'-3')	Use	Comments	Expected product
chiaR	GCCTTGGGGAGAGCCTTGTAGTCC	PCR	Used with chiaF on genomic DNA	931 bp
chiaF	CGGCCGCAACTTCCAGCC	PCR	Used with chiaR on genomic DNA	931 bp
SechiR	CTGGCGGGCACACCACCGGCGATATA	RACE-PCR and RT-PCR	Used with RACE 5' primer on cDNA Used with SechiF	950 bp 592 bp
SechiR nested	CGGCTTCGTTGGCAGGTACTCCCAGTCA	RACE-PCR and RT-PCR	Used with race 5' nested primer on RACE-PCR 5' products	652 bp
SechiF	CGGATATTGAGAAGCACTACCCGACTGA	RACE-PCE and RT-PCR	Used with RACE 3' primer on cDNA Used with SechiR	1365 bp 592 bp
InDanF	CCAGTCATCCATTGCTGCTCC	Sequencing reaction	To obtain the 3'end of *sechi44*	------
InGreR	CCTTCAAACCATGCGGTGCC	Sequencing reaction	To obtain the 3'end of *sechi44*	------
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	PCR	Used with ITS4 on genomic DNA Ref. Gardes and Bruns, 1993	653 bp
ITS4	TCCTCCGCTTATTGATATGC	PCR	Used with ITS1-F on genomic DNA Ref. White et al., 1990	653 bp
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Real-time RT-PCR	Used with Bt2b as internal standard Ref. Glass and Donaldson, 1995	258 bp
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	Real-time RT-PCR	Used with Bt2b as internal standard Ref. Glass and Donaldson, 1995	258 bp

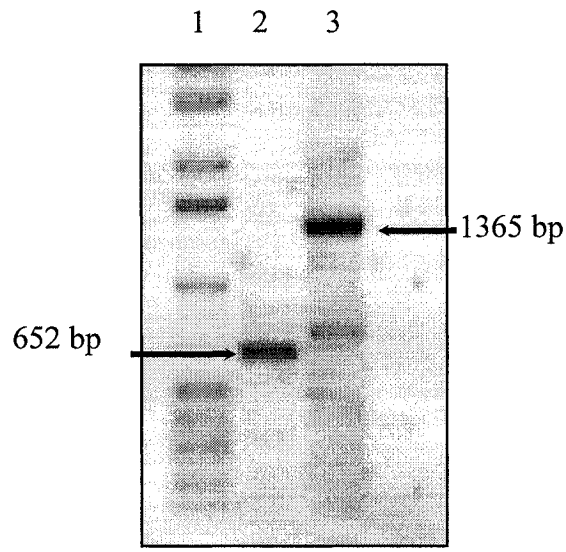


Figure 3.1. RACE-PCR amplifications using *S. elegans* total RNA as template. Total RNA was extracted from mycelium cultured under chitin-rich (induced) conditions. The 5' end (lane 2, 652 bp) and 3' end (lane 3, 1365 bp) of *sechi44* cDNA were amplified with SechiR nested/RACE 5'nested and SechiF/RACE 3' primers, respectively. Ten microliters of each RACE-PCR product were run on a 1% agarose gel. Lane 1 corresponds to the 1 kb DNA ladder molecular weight marker.

Figure 3.2. *S. elegans sechi44* cDNA and deduced amino acid sequences. Boxed amino acid sequences indicate the signal peptide, the chitinase family 18 active site, and the primers used for RACE-PCR and sequencing reactions. The latter is topped by arrows.

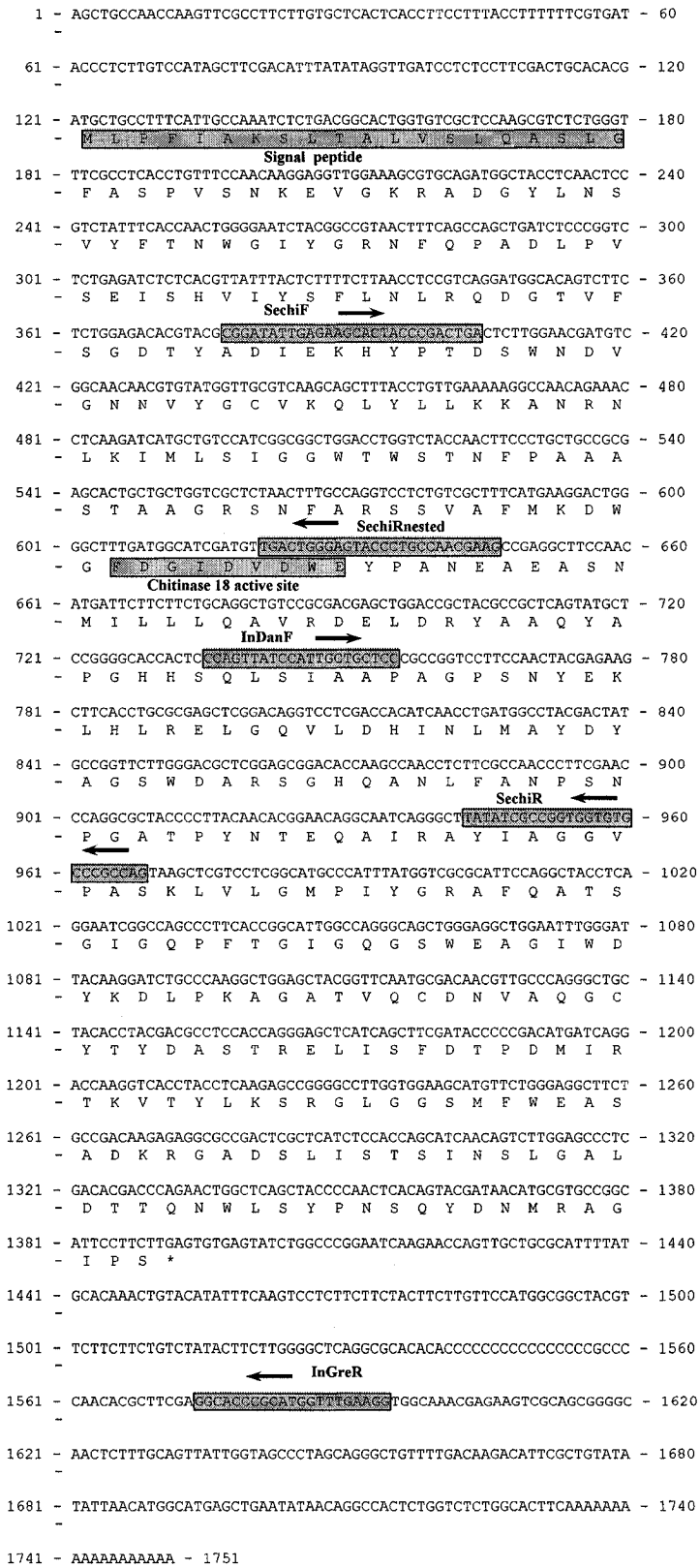

Signal peptide
SechiF
SechiRnested
Chitinase 18 active site
InDanF
SechiR
InGreR

Figure 3.3. Alignment of deduced amino acid sequences of *S. elegans* SeCHI44 with *T. harzianum* CHIT42 (AAB34355), *T. harzianum* CHI1 (AAA98644), *T. atroviride* ECH42 (CAA55928) and *A. album* CHI1 (CAA45468). Identical amino acids with all other sequences indicated by asterisk, with three other sequences indicated by periods, with two other sequences indicated by colon. The alignment was performed with Clustal W (version 1.81).

```
AAB34355        MLSFLGKSVALLAALQATLSSPKP-GHRRASVEKRANGYANSVYFTNWGIYDRNFQPADL 59
AAA98644        MLSFLGKSVALLAALQATLSSASPLATEERSVEKRANGYANSVYFTNWGIYDRNFQPADL 60
CAA55928        MLGFLGKSVALLAALQATLISASPVTANDVSVEKRASGYANAVYFTNWGIYGRNFQPQNL 60
CAA45468        MLSFVKKSIALVAALQAVTALATP-ISSEAGVEKRGSGFANAVYFTNWGIYGRNFQPADL 59
SeCHI44         MLPFIAKSLTALVSLQASLGFASPVSNKE--VGKRADGYLNSVYFTNWGIYGRNFQPADL 58
                ** *  **   .  *** .  .:*    :  * **. *. *:*********:*****..*

AAB34355        VASDVTHVIYSFMNLQADGTVISGDTYADYEKHYADDSWNDVGTNAYGCVKQLFKVKKAN 119
AAA98644        VASDVTHVIYSFMNLQADGTVISGDTYADYEKHYADDSWNDVGTNAYGCVKQLFKVKKAN 120
CAA55928        VASDITHVIYSFMNFQADGTVVSGDAYADYQKHYDDDSWNDVGNNAYGCVKQLFKLKKAN 120
CAA45468        PASEITHVLYSFMNVRADGTIFSGDTYADYEKHYAGDSWNDVGTNAYGCVKQLYLLKKQN 119
SeCHI44         PVSEISHVIYSFLNLRQDGTVFSGDTYADIEKHYPTDSWNDVGNNVYGCVKQLYLLKKAN 118
                  * : **.*** *:  ***. ***.*** .***  ******* * *******  :**.*

AAB34355        RGLKVLLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDIDWEYPADATQA 179
AAA98644        RGLKVLLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDIDWEYPADATQA 180
CAA55928        RNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDVDWEYPADDTQA 180
CAA45468        RNMKVMLSIGGWTWSTNFPAAASSAATRKTFAQSAVGFMKDWGFDGIDIDWEYPADATQA 179
SeCHI44         RNLKIMLSIGGWTWSTNFPAAASTAAGRSNFARSSVAFMKDWGFDGIDVDWEYPANEAEA 178
                *:.* :************** ***. * * .**     *********** ******    *

AAB34355        SNMILLLKEVRSQRDAYAAQYAPGYHFLLTIAAPAGKDNYSKLRLADLGQVLDYINLMAY 239
AAA98644        SNMILLLKEVRSQLDAYAAQYAPGYHFLLTIAAPAGKDNYSNVRLADLGQVLDYINLMAY 240
CAA55928        TNMVLLLKEIRSQLDAYAAQYAPGYHFLLSIAAPAGPEHYSFLHMSDLGQVLDYVNLMAY 240
CAA45468        QNMVLLLQAVRSELDSYAAQYAKGHHFLLSIAAPAGPDNYNKLKFAELGKVLDYINLMAY 239
SeCHI44         SNMILLLQAVRDELDRYAAQYAPGHHSQLSIAAPAGPSNYEKLHLRELGQVLDHINLMAY 238
                :**:***  .*  .* ******.* *  *:******: .* :. :  **.*** .*****

AAB34355        DYAGSFSPLTGHDANLFNNPSNPNATPFNTDSAVKDYINGGVPANKIVLGMPIYGRSFQN 299
AAA98644        DYAGSFSPLTGHDANLFNNPSNPNATPFNTDSAVKDYINGGVPANKIVLGMPIYGRSFQN 300
CAA55928        DYAGSWSSYSGHDANLFANPSNPNSSPYNTDQAIKDYIKGGVPASKIVLGMPIYGRAFES 300
CAA45468        DYAGSWSNYTGHDANIYANPQNPNATPYNTDDAVQAYINGGVPANKIVLGMPIYGRSFQQ 299
SeCHI44         DYAGSWDARSGHQANLFANPSNPGATPYNTEQAIRAYIAGGVPASKLVLGMPIYGRAFQA 298
                *****:    ** **..:**.** ..*:**  *   ** ***** * ********* *.

AAB34355        TAGIGQTYNGVGSGSWEAGIWDYKALPKAGATVQYDSVAKGYYSYNSATKELISFDTPDM 359
AAA98644        TAGIGQTYNGVGSGSWEAGIWDYKALPKAGATVQYDSVAKGYYSYNSATKELISFDTPDM 360
CAA55928        TGGIGQTYSGIGSGSWENGIWDYKVLPKAGATVQYDSVAQAYYSYDPSSKELISFDTPDM 360
CAA45468        TEGIGKPYNGIGSGSWENGIWDYKALPKAGATVKCDDTAKGCYSYDPSTKELISFDTPAM 359
SeCHI44         TSGIGQPFTGIGQGSWEAGIWDYKDLPKAGATVQCDNVAQGCYTYDASTRELISFDTPDM 358
                * ***.   *:* ****:****** ********. * .* . * *: :. ********.*

AAB34355        INTKVAYLKSLGLGGSMFWEASADKKGADSVIGTSHRALGGLDTTQNLLSYPNSKYDNIK 419
AAA98644        INTKVAYLKSLGLGGSMFWEASADKKGADSLIGTSHRALGGLDTTQNLLSYPNSKYDNIK 420
CAA55928        INTKVSYLKNLGLGGSMFWEASADKTGSDSLIGTSHRALGSLDSTQNLLSYPNSQYDNIR 420
CAA45468        ISTKVSWLKGKGLGGTMFWEASASKKGSDSLISTSHQGLGSQDSTQNYLDYPNSKYDNIK 419
SeCHI44         IRTKVTYLKSRGLGGSMFWEASADKRGADSLISTSINSLGALDTTQNWLSYPNSQYDNMR 418
                * *** .**: ****.*******.* *:**.* **   ** .*:*** *.**** ***

AAB34355        NGLN- 423
AAA98644        NGLN- 424
CAA55928        SGLN- 424
CAA45468        KGMN- 423
SeCHI44         AGIPS 423
                  *
```

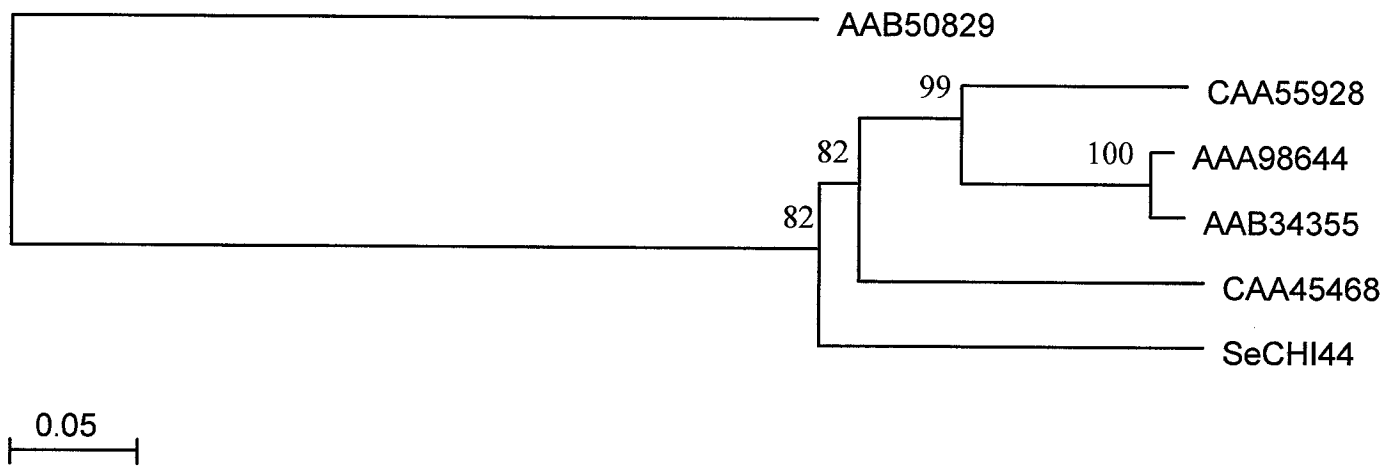
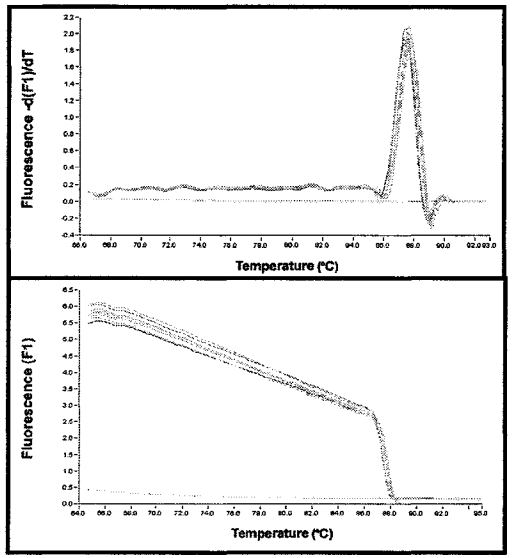
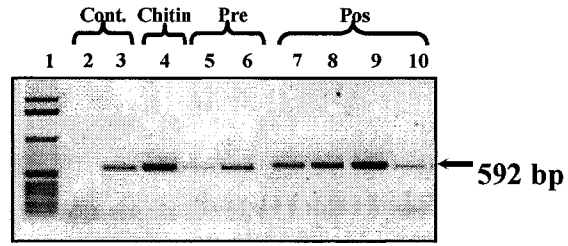


Figure 3.4. Phylogenetic relationships between *S. elegans* endochitinase (SeCHI44) and related chitinase amino acid sequences. The dendrogram was generated by the neighbor-joining method and is rooted to the out-group *T. atroviride* CHIT73 (AAB50829). Bootstrap values (1000 replications) are indicated as percentages at each node. The bar represents 0.05 substitutions per site. CAA55928, *T. atroviride* ECH42; AAA98644, *T. harzianum* CHI1; AAB34355, *T. harzianum* CHIT42; CAA45468, *A. album* CHI1.

Figure 3.5. Analysis of *sechi44* expression under different growth conditions. Expression analysis was performed by RT-PCR and real-time quantitative RT-PCR using SechiF and SechiR primers on total RNA from *S. elegans* and *R. solani* that have been grown on different substrates; a cDNA product of 592 bp was obtained. **(A)** Melting curve profile and corresponding agarose gel electrophoresis for RT- PCR amplification. Lanes 2 and 3 show the results obtained when *R. solani* and *S. elegans*, respectively, grew alone on no carbon (non-induced) source (control). Lane 4 shows the result obtained when *S. elegans* grew on chitin-rich (induced) medium. Lanes 5 and 6 show the results obtained when *S. elegans* and *R. solani* grew on MSMA in dual culture but before contact, when hyphae were distanced by 1.0 and 0.5 cm, respectively ("Pre''-contact). Lane 7, 8, 9, and 10 show results obtained when *S. elegans* and *R. solani* grew on MSMA in dual culture for T_{2d}, T_{3d}, T_{9d}, and T_{12d}, respectively, after contact ("Post"-contact). Ten µl of each RT-PCR product were run on a 1% agarose gel. Lane 1 corresponds to the 1 kb DNA ladder molecular weight marker. **(B)** Quantification of *sechi44* expression using real-time RT-PCR. Kinetics of fluorescence signal measured during induced and non-induced conditions. C_T was determined at the fixed threshold of 0.2. cDNA input was normalized to ascomycete β-tubulin expression measured in parallel PCR reactions. Real-time RT-PCR data are presented as relative expression using $R_{N,b}/R_{N,a} = (1+E)^{-\Delta\Delta C_T}$.

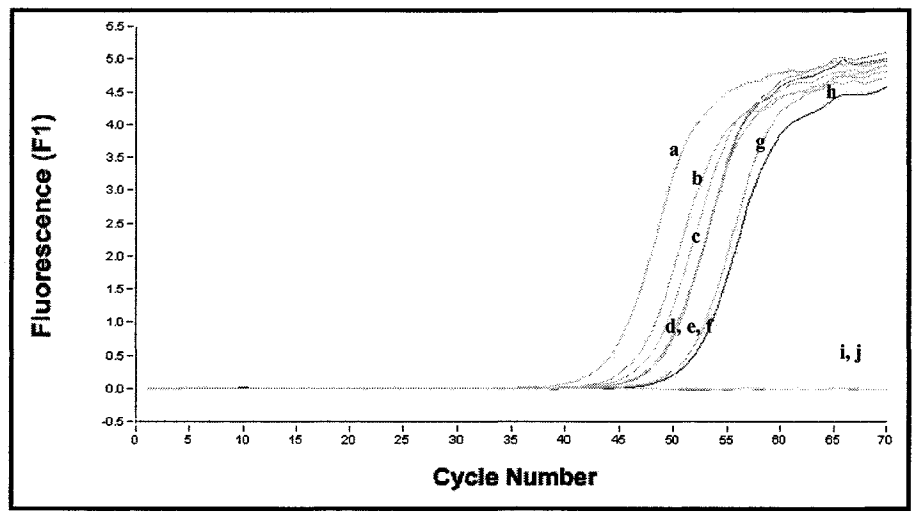
A





B

Curve	Samples	Ratio
f	*S. elegans* alone	1 (control)
g	1.0 cm	0.426 ± 0.079
e	0.5 cm	0.485 ±0.105
d	2 days	0.970 ± 0.370
c	3 days	2.130 ± 0.270
b	9 days	9.955 ± 5.008
h	12 days	0.410 ± 0.359
a	chitin	21.413 ± 12.195
i	*R. solani*	-
j	Negative control	-



3.5. DISCUSSION

S. elegans displays strong mycoparasitic activity against *R. solani* (Benyagoub et al., 1994). The success of *S. elegans* as a potential biological control agent is believed to involve the secretion of CWDEs (Archambault et al., 1998b; Taylor et al., 2002; Tweddell et al., 1995), and the ability of these enzymes to lyse host cell walls and young hyphal tips (Archambault et al., 1998b; Tweddell et al., 1995).

In this study, we report on the cloning and characterization of *sechi44,* a cDNA transcript corresponding to an endochitinase gene encoding a 44-kDa protein from *S. elegans*. The cloning was made possible by RACE-PCR technique that proved to be an efficient and rapid method when compared to cDNA library screening. This method was also successful in cloning an endochitinase gene *chit36* from *T. harzianum* (Viterbo et al., 2001).

The deduced protein sequence of SeCHI44 shows very high identity and similarity with endochitinases of other mycoparasites (Fig. 3.3). Interestingly, the presence of a 20-residue putative signal peptide strongly suggests that SeCHI44 is an extracellular protein, a feature common to the majority of endochitinases expressed by mycoparasites (Hayes et al., 1994; Viterbo et al., 2001). A PSI-Blast search indicated that SeCHI44 belongs to glycosyl hydrolase family 18 class V chitinase (Altschul et al., 1997). Moreover, the phylogenetic tree showed that SeCHI44 is grouped with endochitinases from other mycoparasites. These support our contention that SeCHI44 is an endochitinase as all known mycoparasite endochitinases belong to this family. While limited availability of fungal chitinase sequences makes interpretation of the phylogenetic analysis difficult, SeCHI44 appears to be the most divergent of the fungal chitinase sequences to date.

Southern blot analysis suggested that a single copy of *sechi44* is present in the genome of *S. elegans*, which is consistent with other CWDE-encoding genes of mycoparasites including chitinases, glucanases and protease (Geremia et al., 1993; Hayes et al., 1994; Schaeffer et al., 1994).

Expression of RT-PCR analysis revealed that *sechi44* can be differentially expressed under induced and non-induced conditions (Fig. 3.5). A basal level of

gene expression was detected when *S. elegans* was grown on no carbon (non-induced) medium. Similar results were reported for the endochitinase encoding gene *ech42* of *T. atroviride* (Cortes et al., 1998). In addition, the presence of an external source of carbon such as chitin or the host fungus, *R. solani* appears to be strongly stimulatory to *sechi44* expression. This is consistent with previous studies on chitinase-homologous genes namely *ech42* from *T. atroviride*, (Cortes et al., 1998; Kullnig et al., 2000), *chit42* from *T. harzianum* (Garcia et al., 1994), and *chi1* from *A. album* (Blaiseau et al., 1992).

Although RT-PCR analysis provided evidence that *sechi44* is differentially expressed, comparison of gene expression between chitin-rich medium and during confrontation assays with *R. solani* can not be made, since the retrotranscribed RNA from confrontation assays originated from both the mycoparasite and its host. This may lead to differences in retrotranscription and amplification efficiencies as demonstrated in the band intensity of amplified transcripts when confronting hyphae were 0.5 cm apart (Fig. 3.5A, lane 6). To overcome this limitation, real-time quantitative RT-PCR was conducted in order to accurately estimate the transcription levels of the gene of interest under different induced conditions.

The gradual increase in gene expression after contact followed by a decrease in the expression of *sechi44* after 12 days of contact (Fig. 3.5A, lane 10) in our study is in agreement to what has been reported on endochitinases of *Trichoderma* species during contact (Carsolio et al., 1994; Cortes et al., 1998) affirming that endochitinase expression during mycoparasitism is a general concept. Of interest were the low levels of expression (0.5 fold lower) that were below the basic expression levels in *S. elegans* alone when confronting hyphae were either 1.0 or 0.5 cm apart. This highly suggests that there is some sort of inhibition or a suppression of *sechi44* expression caused by *R. solani*. Whether *R. solani* prior to contact with *S. elegans* produces compounds that play a role in suppression of *sechi44* expression is yet to be determined. Other studies on gene expression of the endochitinase *ech42* before physical contact of *Trichoderma atroviride* with *R. solani* during confrontation assays reported varied results that

ranged from expression to induction (Cortes et al., 1998; Kullnig et al., 2000; Zeilinger et al., 1999). Comparison of our results with these studies cannot justifiably be made, nor can generalization be stated since our study was based on quantitative analysis of gene expression, while theirs were based on qualitative gene analysis involving either GFP fusion gene expression or comparison of band intensities to an internal standard in Northern experiments.

Although a characterization of the full chitinolytic system of *S. elegans* at the gene level is essential to find out which of these enzymes affect pre-contact gene expression and to understand the relevance of this mechanism to biocontrol, we have taken the initial step towards this goal by studying the quantitative expression of the first characterized endochitinase gene of *S. elegans*.

3.6 ACKNOWLEDGMENTS

This study is supported by a Natural Science and Engineering Research Council of Canada (NSERC) operating grant to S. Jabaji-Hare. The authors thank Greg Taylor for his technical assistance throughout the study.

CONNECTING STATEMENT BETWEEN CHAPTERS 3 AND 4

Chapter 3 focused on the cloning and characterization of an endochitinase-encoding gene *sechi44* from *Stachybotrys elegans.* In chapter 4, temporal expression of *sechi44* was quantified under different carbon and nitrogen sources and during the mycoparasitism of *Rhizoctonia solani* by *S. elegans* using QRT-PCR. The influence of various carbon and nitrogen sources on the expression of *sech44* was studied after 6, 24, and 48h of growth in synthetic medium amended with carbon and nitrogen sources. The temporal expression during mycoparasitic interaction was monitored prior to the contact of the mycoparasite with the host's hyphae, at the day of contact, and every day thereafter until twelve days after the hyphae intermingled.

At the time of conducting the experiments described in Chapter 3, statistical programs for evaluating the selection of the best HKGs were not yet developed and thus unavailable. In this Chapter, the software BestKeeper (Pfaffl et al., 2004) was applied to determine which of the HKGs β-tubulin or histone-4, is deemed the most stable and can be used in normalization of gene expression data. Histone-4 was selected and the expression of *sechi44* was normalized relative to histone-4 expression. Quantification of the normalized gene expression was accomplished using the software REST (Pfaffl et al., 2002) and efficiencies were calculated using standard curves.

The results of this section are the subject of a manuscript that is published in the Canadian Journal of Microbiology (2006, Vol. 52: pp. 1-7). I designed the experimental set-up, conducted all the experiments, and wrote the first draft of manuscript. The contributions of the co-authors were as follows: Professor S. Jabaji-Hare provided supervision, funding throughout this study, and made extensive corrections on the manuscript. Professor P. Séguin helped with the choice of statistical methods, analysis of data, and revised the final version of the manuscript.

CHAPTER 4

Expression regulation of the endochitinase-encoding gene *sechi44* from the mycoparasite *Stachybotrys elegans*

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4.1. ABSTRACT

The regulation of the gene encoding the extracellular chitinase *sechi44* produced by the mycoparasite *Stachybotrys elegans* was studied using real-time quantitative reverse-transcription polymerase chain reaction. Alteration of *sechi44* expression was observed when *S. elegans* was in interaction with its host, *Rhizoctonia solani*, and also when the mycoparasite was grown on minimal media amended with different carbon and nitrogen sources. Direct contact with *R. solani* leading to mycoparasitism significantly up-regulated the expression of *sechi44*, although the analysis showed that *sechi44* was constitutively expressed but at substantially lower levels. In addition, the study of *sechi44* over 12 days showed that its expression followed a cyclical pattern with peaks every 2 days, which suggests that this gene has a role not only in mycoparasitism, but also in growth. Addition of external carbon sources such as *N*-acetylglucosamine, chitin, and *R. solani* cell wall (simulated mycoparasitism) triggered an increase in the expression of *sechi44*, which varied with time and carbon source. Among the carbon sources examined, *N*-acetylglucosamine induced the highest increase in *sechi44* transcript levels. Addition of high concentrations of glucose and ammonium triggered a decrease of *sechi44* expression, suggesting that *sechi44* is subject to glucose and ammonium repression.

Keywords: mycoparasitism, *Stachybotrys elegans*, endochitinase-encoding gene, *sechi44*, real-time RT-PCR.

4.2. INTRODUCTION

Many eukaryotic microorganisms degrade chitin, a polymer of *N*-acetylglucosamine, using endochitinases, chitobiosidases, and *β-N*-acetylhexosaminidases (Sahai and Manocha, 1993). Chitinases produced by filamentous fungi have been shown to be involved in a variety of functions such as cell wall digestion, germination of spores, hyphal growth and autolysis, differentiation into spores, assimilation of chitin, and mycoparasitism (Adams, 2004; Flach et al., 1992; Gooday, 1997).

The soilborne fungus *Stachybotrys elegans* (Pidopl.) is reported to produce chitinases (Morissette et al., 2003; Taylor et al., 2002; Tweddell et al., 1995), glucanases (Archambault et al., 1998a), as well as cellulases (Tweddell et al., 1995). As with the genus *Trichoderma*, it displays good mycoparasitic activity with strong capabilities of degrading the cell wall of its host, *Rhizoctonia solani*, and further assimilates and colonizes its cytoplasm (Benyagoub et al., 1994; Benyagoub et al., 1996), making it a good candidate for biological control.

Although a plethora of chitinolytic systems have been detected and purified from several *Trichoderma* isolates (Lorito, 1998), only a limited number of chitinolytic genes, which are specifically induced under mycoparasitic conditions, have been cloned and characterized (*exc1, exc2, ech42, chit42, chit33, nag1, nag2, and chit36*) (Draborg et al., 1995; Garcia et al., 1994; Hayes et al., 1994; Kim et al., 2002; Limon et al., 1995; Viterbo et al., 2001). However, the genetic regulation of these genes is not well understood, and gene expression of only a few have been studied so far on the basis of Northern blot analysis (Brunner et al., 2003b; Dana et al., 2001; Donzelli and Harman, 2001), or using a reporter gene fusion (Zeilinger et al., 1999). For instance, the expression of *ech42* occurs before contact with the host (Zeilinger et al., 1999), is inducible by fungal cell walls and chitin (Carsolio et al., 1999), and is subject to carbon and nitrogen catabolite repression (Donzelli and Harman, 2001; Lorito et al., 1996b). Despite these advances, we still have not observed the total diversity of genes encoding cell wall-degrading enzymes (CWDEs) produced by other mycoparasites and little information is known on the molecular mechanisms that trigger the expression of

CWDEs. A better understanding of the regulation of these genes involved in mycoparasitism could improve the performance of mycoparasites as biocontrol agents, either by predicting their efficiency in complex soil environments or by improving formulation for the spread of the mycoparasite.

Recently, we reported on the cloning of the endochitinase gene *sechi44* from *S. elegans* via the rapid amplification of cDNA ends (RACE)-PCR technique and on its molecular characterization (Morissette et al., 2003). The sequence of this gene shows a high similarity (>80%) with endochitinase genes from *Trichoderma* strains, and its expression occurs during physical contact with its host at different non-continuous time intervals on pooled RNA rather than on true biological replicates (Morissette et al., 2003).

To better understand the factors influencing *sechi44* transcription under conditions relevant to biological control and to know whether its expression is similar to other chitinase-encoding genes of *Trichoderma* species or follows a different pattern, we describe in this study the temporal alteration of *sechi44* expression under different carbon and nitrogen sources using quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis. We also studied the expression of *sechi44* every 24 hours over a period of 12 days of mycoparasitism. We chose to apply QRT-PCR over the Northern blot technique because it is a powerful tool for the detection and quantification of relative amounts of mRNA; is highly sensitive, accurate and reproducible; and does not require large amounts of RNA, thus allowing the examination of several biological replicates simultaneously with the possibility to conduct robust statistical analysis (Pfaffl et al., 2002), which previous studies could not perform.

4.3. MATERIALS AND METHODS

4.3.1 Fungal strains and growth conditions

Starter cultures of *S. elegans* (ATCC 188825, anamorph), and *R. solani* AG-3 (ATCC 10183), the anamorph of *Thanatephorus cucumeris,* were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich., USA) at 24°C for 7 days and 5 days, respectively.

4.3.2 Cell wall preparation

Purified *R. solani* cell wall fragments were prepared as described in Tweddell et al. (1994). Agar plugs (6 mm) from a starter culture of *R. solani* AG-3 were used to inoculate 100 ml of potato dextrose broth (Difco) at 24°C for 7 days with agitation at 110 rpm. The mycelium was collected through Whatman No.1 filter paper, washed with sterile water, homogenized, and treated in an ultrasonic disintegrator for 10 min. Cell wall fragments were centrifuged twice (160 *g.* and 16,000 *g.*) for 10 min and then washed with sterile water until no residual glucose and proteins could be detected in the supernatant. Glucose and protein concentrations were determined with the glucose oxidase reagent and by the Bradford method, respectively (Bradford, 1976). Freeze-dried purified cell wall fragments were ground into a fine powder with a mortar and pestle and autoclaved at 121°C for 15 min.

4.3.3 Expression of *sechi44* under carbon and nitrogen amendment

Agar plugs (6 mm) from 5-day old starter cultures of *S. elegans* were used to inoculate 15 ml of minimal synthetic medium (MSM; (Tweddell et al., 1995) amended with 1.5% *m/v* glucose (Sigma, St.Louis, Mo., USA) in a 100 mm diameter Petri plate and were left to grow without agitation in the dark at 24°C for 3 days. The young mycelia were washed twice with sterile deionized water, and excess water was removed by aspiration. The washed mycelia were resuspended in 10 ml of MSM (pH 5.2) that was supplemented with the following carbon sources: colloidal chitin (1 g/L, Sigma), laminarin (5 g/L, Sigma), glucose (2 and 20 g/L), glycerol (2% v/v, Anachemia, Montréal, Canada), *N*-acetylglucosamine (GlcNAc, 1 g/L, Sigma), purified *R. solani* cell wall fragments (1 g/L) or with NH_4NO_3 (0.12 mmol or 12 mmol) as the nitrogen source. Mycelia resuspended in MSM not amended with carbon or nitrogen sources were used as the control. Triplicate plates for each carbon and nitrogen source (induced conditions) and the control treatment (non-induced, starved condition) were incubated in the dark for 6, 24, and 48 h, after which the mycelia were collected insuring that all of the MSM had been removed, either by aspiration or by blotting excess MSM using

sterile Whatman paper. Harvested mycelia were immediately flash frozen in liquid nitrogen and stored at -80°C.

4.3.4 Expression of *sechi44* during dual interaction assays

For confrontation studies, agar plugs (6 mm) from starter cultures of *S. elegans* and *R. solani* were placed on fresh potato dextrose agar in different Petri plates (100 mm diameter), and mycelia were allowed to grow for 5 days. Plate confrontation assays were carried out in the absence of light on culture plates containing MSM supplemented with 1% *m/v* agar (MSMA; Gellan Gum, Kelco, San Diego, Calif., USA) and covered with a permeable cellophane membrane (500 PUT; UCB, North Augusta, S.C., USA). Agar plugs (6 mm) from the respective fungi were placed on the surface of the cellophane membrane 6 cm from each other. This set-up allowed the mycoparasite and its host to grow towards and contact each other, and to eventually interact with each other, a condition that we refer to as induced. In addition, the use of the membrane facilitated the removal of the fungi from the plate for subsequent RNA analysis. Total RNA from triplicate plates was extracted from (i) hyphae of both fungi when they were distanced 0.5 cm apart prior to contact, (ii) a 5 cm strip of both fungi at the zone of interaction every 24 h from the day of contact (day 0) until day 12, and (iii) hyphae of pure cultures of *S. elegans* grown on MSMA alone without its host, and harvested at the same time periods (i.e., non-induced condition or control). The mycelia from all treatments and time periods were flash frozen in liquid nitrogen as they were harvested and stored at -80°C.

4.3.5 Primer Design

The gene-specific primer pair sec1F and sec1R was designed using DNAman software (version 4.13; Lynnon Corporation, Vaudreuil-Dorion, Canada), based on the sequence of the endochitinase-encoding gene *sechi44* (accession number AF516397). The gene *sechi44* has a 1269-bp open reading frame, and the primers sec1F (GTACGCGGATATTGAGAAGCACTAC) and sec1R (TGGACAGCATGATCTTGAGGTTT) were designed to amplify an

amplicon of 128 bp, from position 371 to position 499, based on the cDNA sequence. Two housekeeping genes (HKGs) were used in this study; the two primer sets, Bt2a and Bt2b and H4-1a and H4-1b, were designed by Glass and Donaldson (1995) to amplify a β-tubulin-encoding gene segment of 260 bp in length, and a histone-4-encoding gene segment of 161 bp in length from Ascomycetes. The specificity of all the primers was checked by alignment with GenBank sequences using the standard nucleotide-nucleotide BLAST (blastn; (Altschul et al., 1997). Conventional reverse transcription (RT)-PCR assays followed by gel electrophoresis were performed to verify amplification of cDNA using the designed primers. Melting point analysis was performed at the end of the real-time PCR to confirm the amplification of a unique product for the target gene and the HKGs.

4.3.6 Isolation of RNA and reverse transcription

Frozen mycelia were ground into a fine powder in liquid nitrogen using a mortar and pestle. Total RNA from 100 mg of powder was isolated using the RNeasy Plant Mini Kit™ (QIAGEN, Mississauga, Ont., Canada) and treated with RNase-free DNase I™ (QIAGEN) according to the manufacturer's recommendations. Contamination of RNA samples with DNA was verified by PCR amplification of total RNA with H3-1a and H3-1b primer set (glass and Donaldson 1995), which amplifies a segment of the histone-3-encoding gene from Ascomycetes. The concentration and purity of RNA were checked by absorbances at 260 nm and 280 nm, while the quality of RNA was checked on 1% *m/v* formaldehyde agarose gel. Total RNA amounts of 500 ng (from interaction assay) and 200 ng (from C and N treatments) were reverse transcribed using the Omniscript Kit™ (QIAGEN), following the manufacturer's recommendations.

4.3.7 Standard curve construction

Conventional RT-PCR assays were performed on cDNA templates prepared from total RNA that was extracted from *S. elegans* grown on MSM, using the sec1F and sec1R and the HKG primers. cDNA templates were diluted

25-fold and amplification was performed in a 25 µl reaction containing 1x PCR buffer (Invitrogen, Burlington, Ont., Canada), 0.2 mmol dNTPs, 200 nmol primers, 1.0 mmol $MgCl_2$, 2 U Taq DNA polymerase (Invitrogen), and 2 µl of diluted cDNA. In all RT-PCR runs, appropriate negative controls containing water rather than cDNA template, were subjected to the same procedure to exclude or detect possible contamination or carryover. The RT-PCR runs consisted of an initial denaturation at 93°C for 3 min, 35 cycles of 1 min at 95°C, 1 min at 58°C, 2 min at 72°C, and an extension cycle at 72°C for 10 min.

The RT-PCR products for the target gene and the HKGs were purified using a QIAquick PCR Purification Kit™ (QIAGEN) and quantified on an agarose gel with a low molecular weight ladder (Invitrogen). Depending on the gene, serial dilutions of purified RT-PCR products in the range of 0.12 fg to 1.2 ng and over five-fold or six-fold orders of magnitude were prepared to generate standard curves for real-time QRT-PCR assays. Each standard curve was run in duplicate, and four technical replicates were done for each run. The efficiency of amplification was calculated by averaging all the replicates.

4.3.8 Expression analysis of *sechi44* by real-time QRT-PCR.

Real-time QRT-PCR was performed, for the target gene *sechi44* and for the two HKGs (β-tubulin and histone-4), using the Mx3000™ (Stratagene, Cedar Creek, Tex., USA) and the SYBR Green Master Mix™ (Stratagene) following the manufacturer's recommendations. Amplification was performed in a 24 µl reaction mixture containing the following concentrations: 125 nmol for each primer, 1x SYBR Green Master Mix™, 30 nmol for the reference dye ROX, and 4 µl of cDNA template corresponding to 4 ng of total RNA (corresponding to 1/25 of the interaction samples and 1/10 of the total cDNA from C and N samples). The amplification conditions were 95°C for 10 min (hot start); followed by 45 cycles of 95°C for 30 s, 1 min at 68, 61, and 66°C for *sechi44*, histone-4, and β-tubulin, respectively; and 72°C for 30, 20, and 30 s for *sechi44*, histone-4, and β-tubulin, respectively. Following amplification, a melting curve program (55-95°C with a heating rate of 0.1°C/s) was added. The reading of fluorescence

was done for 11 s at 78, 81, and 82 °C for *sechi44*, histone-4, and β-tubulin, respectively.

4.3.9 Data quantification

Quantification was based on a 128-bp generated amplicon using the gene-specific primer pair sec1F and sec1R. The relative expression of *sechi44* was normalized against the expression of the best HKG that exhibited minimal variation across treatments using the statistical software tool Bestkeeper (Pfaffl 2004); http://www.gene-quantification.info/).

Data generated by real-time QRT-PCR were estimated using Stratagene analysis software. QRT-PCR reaction for each sample was repeated twice (technical replicates). Real-time QRT-PCR data were calculated as a relative expression of gene expression using the software REST™ (Pfaffl 2004; http://www.gene-quantification.info/) and presented as $\log_2$ ratio. The software calculates the relative expression ratio of the target gene on the basis of the PCR efficiency (E) and crossing point difference (ΔCP), and on a newly developed pair-wise fixed reallocation randomization test. The statistical test was calculated at $P = 0.05$ and 2000 randomizations were performed. The relative expression ratios of the endochitinase-encoding gene *sechi44* under induced conditions versus the control (i.e. non-induced condition) were calculated using equation [4.1], in which E is the PCR efficiency calculated from the slope according to the equation $E=10^{(-1/slope)}$; E_{target} is the real-time RT-PCR efficiency of the endochitinase-encoding gene *sechi44*; E_{ref} is the real-time RT-PCR efficiency of the HKG; ΔCP_{target} is the crossing point (CP) deviation of non-induced (control) – induced of *sechi44*; ΔCP_{ref} is the CP deviation of non-induced (control) – induced of the HKG.

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP_{target}\,(\text{MEAN control} - \text{MEAN sample})}}{(E_{ref})^{\Delta CP_{ref}\,(\text{MEAN control} - \text{MEAN sample})}} \qquad [4.1]$$

The target gene was considered differentially expressed compared to the control, if the change of expression was over two fold (i.e. $\log_2 > 1$; Pfaffl et al. 2002).

4.4. RESULTS

4.4.1. Validation of reference gene for normalization

Compared to β-tubulin, the expression levels obtained for histone-4-encoding gene exhibited the lowest variation with a standard variation (SD) <1 and a coefficient of variation (CV) of 2.46 (for the carbon and nitrogen sources experiment) and 2.32 (for the dual interaction experiment). Because of this low variation in expression, the HKG histone-4 could be considered stable. There was no correlation between the two HKGs (coefficient of correlation r = 0.322 and P = 0.083 for the carbon and nitrogen sources experiment, and r = 0.227 and P = 0.245 for the dual interaction experiment). Thus, the relative expression ratio of the endochitinase-encoding gene *sechi44* when *S. elegans* was growing under different carbon and nitrogen sources and during dual interaction was estimated by normalizing it against histone-4.

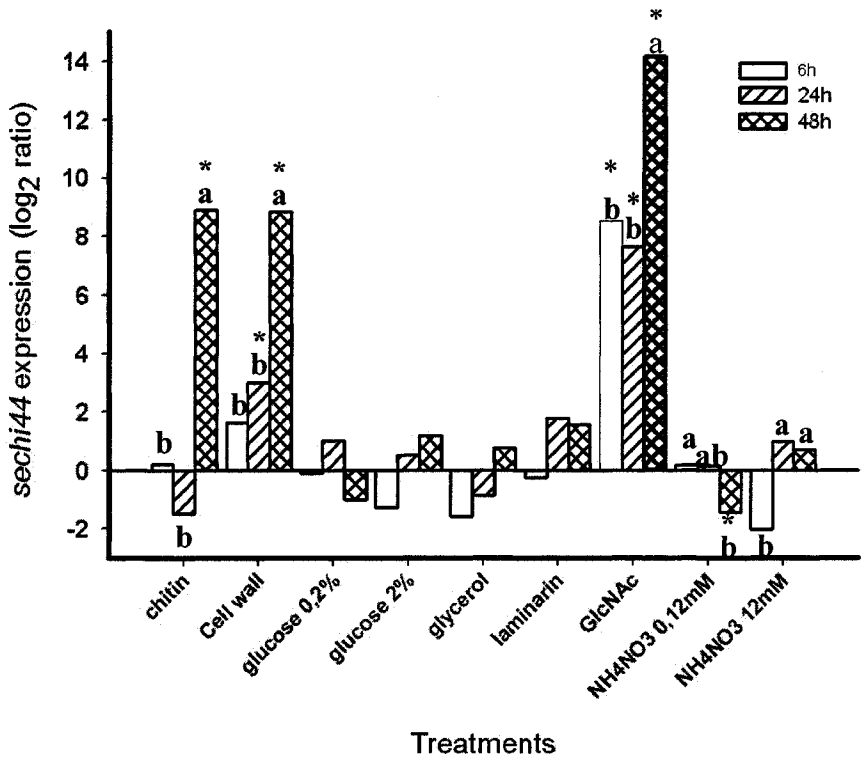
4.4.2. Expression of *sechi44* under different carbon and nitrogen amendment

Under induced conditions, the relative expression of *sechi44* varied (P < 0.05) with different carbon sources and incubation periods (Fig. 4.1). Compared to non-induced conditions, a strong *sechi44* expression was detected as early as 6 h when *S. elegans* was grown on GlcNAc, reaching a significant (P = 0.033) and substantial increase (>16 000-fold) after 48 h. The same temporal pattern of gene induction was observed in the presence of cell wall (simulated mycoparasitism), but the expression level was considerably lower (500-fold) after 48 h. In the presence of chitin, expression of *sechi44* (500-fold) after 48h (P = 0.059) were also observed. Compared to GlcNAc, cell wall, and chitin, substantially lower levels of expression were observed in the presence of laminarin, glycerol, and glucose (0.2% and 2%), as well as in the presence of a nitrogen source (NH_4NO_3; 0.12 or 12mmol). During certain incubation periods and carbon and nitrogen sources, the expression levels of *sechi44*, although not significant, were almost

two- to four-fold lower than the levels estimated when *S. elegans* was grown in the absence of carbon and nitrogen sources (Fig. 4.1).

4.4.3. Expression of *sechi44* during mycoparasitism

For analysis of *sechi44* expression during mycoparasitism, real-time QRT-PCR was performed on cDNA from total RNA of dual cultures of *S. elegans* and *R. solani* over a period of 12 days. The relative expression of *sechi44* followed a cyclical pattern with peaks every 2 days, reaching eight-fold ($P = 0.025$) in the presence of its host 4 days after interaction (Fig. 4.2). Interestingly, before physical contact in the confrontation assay with *R. solani*, almost four-fold lower levels of *sechi44* than the control were observed. When *S. elegans* was grown alone on MSM without the presence of the host (starved conditions), *sechi44* was expressed but at substantially lower levels, and its temporal expression had a similar cyclical pattern (data not shown).



Log (to base 2) ratio difference	Fold ratio
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
11	2048
12	4096
13	8192
14	16384

Figure 4.1. Temporal expression of *sechi44* when *Stachybotrys elegans* is grown under different carbon and nitrogen sources. The data were normalized with the expression of the housekeeping gene, histone-4. The y axis is the log2 ratio of the expression of *sechi44* during induced conditions. The ratios were calculated based on the software REST™ (Pfaffl 2002). Asterisks above the bars indicate significant differences of *sechi44* expression compared with that of the control ($P < 0.1$). Letters above the bars indicate significant differences of *sechi44* expression between the time periods ($P < 0.05$); GlcNAc, *N*-acetylglucosamine.

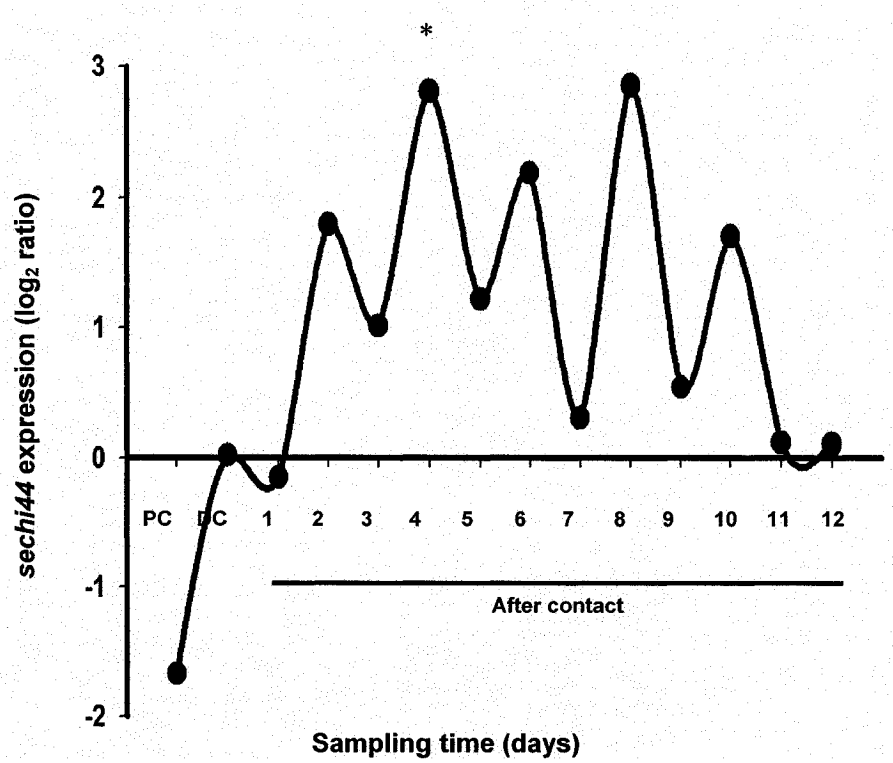


Figure 4.2. Temporal expression of the endochitinase-encoding gene, *sechi44*, during mycoparasitism. The data were normalized with the expression of the housekeeping gene, histone-4. The y axis is the log2 ratio of the expression of *sechi44* during mycoparasitism. The ratios were calculated based on the software REST™ (Pfaffl 2002). The x axis is the time period of interaction. PC, RNA was extracted from mycelia prior contact of the hyphae; DC, RNA was extracted from mycelia at the day of contact. Asterisk indicates significant differences of *sechi44* expression compared with that of the control ($P < 0.05$).

4.5. DISCUSSION

The QRT-PCR-based technique was applied in this study to accurately quantify the levels of temporal alteration of *sechi44* transcripts, an endochitinase, of the mycoparasite *S. elegans* under induced conditions and during mycoparasitism. We chose this method over Northern blotting as a quantification method for fungal gene expression, as it is more accurate and sensitive. Northern blotting can be problematic if the target gene has a low level of expression, as is the case when studying mycoparasitic interaction, or if a limited amount of tissue is available for RNA extraction, thus limiting the presence of biological replication and preventing statistical analysis of data. A PCR-based technique, such as the real-time QRT-PCR, can alleviate these problems if certain factors are appropriately controlled such as well designed primers, standard curves with coefficient correlations (r^2) values close to 1.0, a stable HKG (Pfaffl et al., 2004), and the application of a proper mathematical model (Pfaffl et al., 2002) that accounts for both differences in amplification efficiency and threshold cycle numbers.

Expression of the majority of the chitinolytic genes encoding CWDEs in mycoparasitic fungi can be induced by fungal cell wall extracts, polymers such as chitin, or chitin oligomers (Donzelli and Harman, 2001; Haran et al., 1996; Peterbauer et al., 1996; Viterbo et al., 2002a), although induction of gene expression by chitin has not been consistent, such as in the case of *ech42* (Mach et al., 1999; Margolles-Clark et al., 1996). In agreement with these studies, we found that *sechi44* expression can be triggered, although with different efficacies depending on the nature of the carbon source present in the culture medium. The addition of a readily available external carbon source such as GlcNAc resulted in a strong induction of *sechi44.* Cell wall fractions (simulated mycoparasitism) and chitin, two complex carbon sources, also result in an induction but to a lesser degree. The temporal difference in *sechi44* induction between readily and non-readily available carbon sources, such as chitin and fungal cell wall, may be explained by the fact that a minimum time is required for the enzyme to hydrolyze complex carbon sources releasing readily available oligomers. Recently, Hoell et

al. (2005), showed that chitin requires at least 24 h to be hydrolyzed by the endochitinase Ech30 from *T. atroviride*, releasing monomers that in turn act as inducers. Similarly, significantly higher activity of another endochitinase CHT42 from *T. virens* grown on *R. solani* cell walls, was reported after 48 h (Beak et al., 1999).

Physiological stress and carbon and nitrogen starvation also appear to be involved in the expression alteration of some of the genes encoding chitinases (Dana et al., 2001; Mach et al., 1999; Viterbo et al., 2002a) and glucanases (Lora et al., 1995). In this study, QRT-PCR analysis revealed that the basal level of *sechi44* expression can be detected when *S. elegans* is grown in the absence of a carbon source (non-induced or starvation conditions). Similar results were reported for the *T. harzianum* endochitinase encoding genes *ech42* (Carsolio et al., 1994; Cortes et al., 1998; Lu et al., 2004) and *nag1* (Peterbauer et al., 1996) and at the protein levels for CHIT42 (Limon et al., 1995) and CHIT102 (Haran et al., 1996).

Interestingly, the sensitivity of the QRT-PCR technique enabled us to detect the repression of *sechi44* expression at 6 h in the presence of a high concentration of either glucose or nitrogen, which suggests a glucose and ammonium repression. These results would have been interpreted as an absence of expression if the same data were to be analyzed by Northern blotting. No RNA transcript would have appeared, similar to the results shown for *chit33 and ech42* endochitinase genes, when *T. harzianum* was grown on glucose (Carsolio et al., 1994; Dana et al., 2001), and for *ech42* and *chit36* when *T. atroviride* was grown on a high concentration of ammonium (Viterbo et al., 2002a).

Direct confrontation assays are a powerful tool to study the phenomenon of mycoparasitism by antagonists (Cortes et al., 1998; Kullnig et al., 2000; Zeilinger et al., 1999). However, only a few studies have reported on the expression of genes encoding CWDEs during *in vivo* interaction, and the evidence on the expression of these genes during direct confrontation assays has produced contradictory results. In the case of *T. atroviride*, the chitinase gene *ech42* and the cell wall proteinase gene *prb1* are strongly expressed before physical contact and

during confrontation with *R. solani* (Carsolio et al., 1994; Cortes et al., 1998; Kullnig et al., 2000; Zeilinger et al., 1999), while *nag1* (Zeilinger et al., 1999) and *chit33* (Dana et al., 2001) are expressed only after physical contact. In our direct confrontation study, we were able to follow the alteration of *sechi44* expression every 24 h over a period of 12 days, an extensive period over from what we have previously published (Morissette et al., 2003). We show that, in response to the presence of the host, there is a substantial induction of *sechi44* after 4 days of physical interaction. This period coincides with the time in which the mycoparasite penetrates and colonizes living cells of *R. solani* (Benyagoub et al., 1994) and is in its exponential growth period (Appendix II). At later days of contact, *sechi44* is expressed but the levels are comparable to those when *S. elegans* is alone. It is hard to explain why the expression of *sechi44* would follow a cyclical pattern with an up-regulation every 2 days, but it could be related to vegetative mycelial growth and hyphal branch formation. In addition to the role of chitinolytic enzymes of mycoparasites to degrade the chitin-containing cell wall of other fungi (Haran et al., 1996; Markovitch and Kononova, 2003), fungal chitinases are also important for the survival of the producing organism itself because they are involved in important morphogenetic processes, such as spore germination and hyphal elongation and branching (Sahai and Manocha, 1993). (Dryesen and Nielsen, 2003) suggested that branch formation is directly linked to nuclear division that follows a cyclical pattern. Although more studies are required, it is tempting to hypothesize that if *sechi44* is involved in hyphal branching, the cyclical expression pattern observed in the presence and absence of a host may be synchronized with mitosis.

Together, the results of this study and that of our previous study (Morissette et al., 2003) demonstrated that there is a repression, although not significant, in *sechi44* expression before both fungi make contact. We are not able to explain these results, but one possible hypothesis that merits pursuing is that *R. solani*, in response to the presence of *S. elegans*, produces an inhibitor shortly after exposure to the mycoparasite. Light microscope observations of a parallel study that we are currently conducting on mycoparasitism, in which

conidia of *S. elegans* are sprayed on actively growing cultures of *R. solani*, clearly indicate that conidial germination is substantially delayed for 30 h compared with when conidia are sprayed on *S. elegans* cultures or on culture media without a carbon source.

In conclusion, we demonstrate that the expression of *sechi44* is substantially induced by the presence of GlcNAc, chitin, and *R. solani* cell wall and is repressed by high concentrations of carbon and ammonium. We also demonstrate for the first time that the expression of the endochitinase gene follows a cyclical pattern, and it is involved in both growth and mycoparasitism. Whether the expression of *sechi44* in both events is regulated by a common or different factor remains to be determined.

4.6. ACKNOWLEDGEMENTS

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CONNECTING STATEMENT BETWEEN CHAPTERS 4 AND 5

In chapters 3 and 4, the endochitinase-encoding gene *sechi44* expressed by the mycoparasite *S. elegans* has been cloned and its expression has been studied. The results of these studies suggested that this gene is involved not only in mycoparasitism but in linear growth as well. In order to identify other genes which are involved in the mycoparasitic process, a large scale EST study was conducted and is described in this chapter. Several ESTs were isolated by means of a subtracted cDNA library. This library contained cDNAs expressed only during the mycoparasitic interaction of *S. elegans/R. solani*, and not when the two fungi were grown alone. A total of 120 unique ESTs were isolated and assigned to putative functions. These ESTs represented genes that were preferentially expressed either from *S. elegans* or *R. solani* during their interaction.

The results of this section will be resubmitted for publication in Current Genetics. I have designed the experimental set-up, conducted all the experiments, and wrote the manuscript. The contributions of the co-authors were as follows: Professor S. Jabaji-Hare provided supervision, and funding throughout this study. She also made suggestions and corrected several versions of the manuscript. Dr. Amélie Dauch, helped in several technical aspects and commented on the final version of the manuscript. Drs Roland Brousseau and Luke Masson provided technical advice and guidance in the microarray section and revised the final version of the manuscript.

CHAPTER 5

Transcriptome analysis during interaction of the mycoparasite *Stachybotrys elegans* with its host *Rhizoctonia solani*

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5.1. ABSTRACT

The aim of this study was to identify genes that are up-regulated during mycoparasitism either from the mycoparasite *Stachybotrys elegans* or its host *Rhizoctonia solani*. Suppression subtractive hybridization (SSH) was used to create a subtracted cDNA library, and microarray analysis to identify the overexpressed transcripts. We report the analysis of 2166 clones, among which 47% of the the inserted genes were up-regulated during mycoparasitism. Two hundred sixty-one clones were sequenced and correspond to 120 unique expressed sequence tags (ESTs). Forty-three of these ESTs were identified as novel genes, while the remainer showed similarity to a broad diversity of genes with putative functions related to metabolism, toxin production and pathogenicity. As a result of mycoparasitism, 15 ESTs were identified in *R. solani* whose functions were related to defense. To validate the microarray data, the overexpression of 13 selected genes were confirmed using Quantitative real-time Reverse Transcription (QRT)-PCR.

Index descriptors: mycoparasitism, SSH, microarrays, gene expression, mycoparasitism-related genes, ESTs.

5.2. INTRODUCTION

Stachybotrys elegans is a typical mycoparasite that colonizes its host, *Rhizoctonia solani*, by accomplishing several successive steps: recognition and production of fimbrial extracellular matrix that surrounds the host cell (Benyagoub et al., 1994; Benyagoub et al., 1996), coiling and the formation of appressoria that aid in penetrating the host cell wall followed by intracellular colonization (Benyagoub et al., 1994). This process is accompanied by the secretion of cell wall-degrading enzymes (CWDEs) including, chitinases (Morissette et al., 2003; Taylor et al., 2002; Tweddell et al., 1995), glucanases (Archambault et al., 1998a), and cellulases (Tweddell et al., 1995). As with *Trichoderma* species (for review see Herrera-Estrella and Chet, 2004), the CWDEs of *S. elegans* are released into culture medium amended with purified cell walls of *R. solani* or with chitin as carbon source (Archambault et al., 1998b). An endochitinase gene *sechi44* was cloned and characterized (Morissette et al., 2003), and evidence for its participation in the mycoparasitic process, its stimulation by purified host cell wall fragments, and its regulation by nitrogen and carbon availability was recently provided (Morissette et al., 2006).

In comparison to what has been published on the isolation, characterization and activity of CWDEs and their encoding genes of *Trichoderma* spp (Benitez et al., 2004; Viterbo et al., 2002b), and other mycoparasites (Giczey et al., 2001; Kumar et al., 2000; Mathivanan et al., 1998; McQuilken and Gemmell, 2004; Morissette et al., 2003), little is known about the involvement of non-CWDE genes and their role in mycoparasitism. Various proteins including extracellular proteases and/or genes encoding for serine and aspartic proteases in strains of *T. harzianum*, *T. virens*, and *T. atroviride* (Delgado-Jarana et al., 2002; Grinyer et al., 2005; Pozo et al., 2004; Suarez et al., 2005), G-protein (Omero et al., 1999; Rocha-Ramirez et al., 2002), non-ribosomal peptide synthetase *Tex1* (Wiest et al., 2002), and squalene epoxidase (Cardoza et al., 2006) have been characterized and shown to be implicated in mycoparasitism. The secretion of these proteins and the expression of their encoding genes were conducted using non-induced conditions or artificially induced culture media containing chitin or

purified fungal cell walls as carbon source. Recently, a global analysis of gene expression in mycelium of *T. harzianum* was published using expressed sequence tag (EST) approach (Liu and Yang, 2005). However, the analysis of the identified clones did not reflect the genetic regulation of the mycoparasitic interaction with a living host and its defense reaction, but was rather the result of gene expression in the presence of cell wall preparation. Thus, it is not surprising that among the genes identified several were related to the hydrolysis of fungal cell walls.

The identification of genes presumably involved during the mycoparasite-host interaction was recently analyzed. Carpenter et al. (2005) applied suppression subtractive hybridization (SSH) and explored differential gene expression of the mycoparasite *T. harzianum* in the presence of the living host *Sclerotinia sclerotiorum*. Unfortunately, only a limited subset of novel genes were identified (a total of 19) resulting in an incomplete view of genetic regulation during mycoparasitism. Except for this recent finding, differential expression studies on mycoparasites other than *Trichoderma* in the presence of live hosts have not been reported to our knowledge.

Our aim in this work was to contribute to the wider picture of mycoparasitism, by characterizing genes preferentially expressed during the mycoparasitic interaction between *S. elegans* and *R. solani*. Our approach in this study was to generate a subtracted EST library comprised of 2166 anonymous cDNA clones and identify genes differentially expressed just prior to, and at early and late stages of mycoparasitism using microarray analysis, to confirm the differential expression of selected ESTs using quantitative real-time reverse-transcription (QRT)-PCR, and to annotate ESTs according to their protein functions. This study provides an insight into the transcriptomes that drive the mycoparasitic process in *S. elegans*.

5.3. MATERIALS AND METHODS

5.3.1. Fungal strains and plate confrontation assays

Starter cultures of *S. elegans* (Pidoplichko) W. Gams (anamorph; ATCC 188825) and *R. solani* Kuhn AG-3 (ATCC 10183), the anamorph of

Thanatephorus cucumeris (A.B. Frank) Donk, were revived on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) at 24 °C for 7 and 5 days, respectively. Agar plugs (6 mm) from starter cultures of *S. elegans* and *R. solani* were placed on fresh PDA in different Petri plates (100 mm), and allowed to grow for 5 days.

Plate confrontation assays were carried out in the absence of light on culture plates containing minimal synthetic medium (Tweddell et al., 1995) supplemented with 1% agar (MSMA; Gellan Gum, Kelco, San Diego, USA) and covered with a permeable cellophane membrane (500 PUT; UCB, North Augusta, USA). Agar plugs (6 mm) from the respective fungi were placed on the surface of the cellophane membrane by 6 cm from each other. This set-up allowed the mycoparasite and its host to grow towards and contact each other, and eventually interact with each other. The zone where contact occurs is referred to as the zone of interaction. In addition, the use of the membrane in this set-up facilitated the removal of the fungi from the plate for subsequent RNA analysis. Total RNA from triplicate plates was extracted from (i) hyphae of both fungi when they were distanced 0.5-cm apart prior to contact, (ii) a 5-cm strip of both fungi at the zone of interaction every 48 h from the day of contact (day 0) until 12 days, and (iii) hyphae of pure cultures of *S. elegans* and *R. solani* grown alone, without interaction, on MSMA and harvested at the same time periods (control). The mycelia from all treatments and time periods were flash frozen in liquid nitrogen as they were harvested and stored at -80 °C.

5.3.2. Isolation of genomic DNA, nuclear ribosomal DNA, and RNA

Genomic DNA (gDNA) from *S. elegans* and *R. solani* were extracted as described in Morissette et al. (2003). DNA quality and concentration were checked by electrophoresis using 1% 1x TAE agarose gel and a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE).

Nuclear ribosomal DNA (nrDNA) from both organisms were amplified by PCR using the primer sets LR7R/LR12 and LR0R/LR7 which amplify the large subunit, NS1/NS8 which amplify the small subunit, and ITS4/ITS5 which

amplifies the internal transcribed sequence (ITS) region (Table 5.1). All amplicons were sequenced (Genome Quebec, Montreal, Canada to confirm their identity (GenBank accession nos. DQ369855 to DQ369862; Appendix I), then pooled together (20 µl), purified using phenol-chloroform precipitation, and resuspended in 21 µl. The purified nrDNAs were included in the printed subtracted library in order to evaluate the redundancy of nuclear ribosomal DNA.

Frozen mycelia harvested from the dual interaction experiment were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA from 100 mg of powder was isolated using the RNeasy Plant Mini Kit (QIAGEN, Mississauga, Canada) and treated with RNase-free DNase I (QIAGEN) according to the manufacturer's recommendations. Contamination of RNA samples with DNA was verified by PCR amplification of total RNA with the H3-1a and H3-1b primer set which amplifies a segment of the histone-3-encoding gene from ascomycetes, and with the SBU177 and SBL336 primer set which amplifies a segment of the β-tubulin-encoding gene from basidiomycetes (Table 5.1). The integrity and quality of RNA samples were checked by their absorbance at 260 and 280 nm and by 1% formaldehyde agarose gel. One µg of RNA from the eight time points were pooled (Fig. 5.1), precipitated, and resuspended to a final concentration of 1 µg/µl of RNA mixture. Tester and driver cDNA (Fig. 5.1) were synthesized from 1 µg of RNA using the SMART^{TM} cDNA synthesis kit (BD Biosciences-Clontech, Palo Alto, CA).

5.3.3. Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) was performed between the cDNA populations of the tester (T: zone of interaction between *S. elegans* and *R. solani*.) and the driver (D: *S. elegans* and *R. solani* growing on different Petri plates, control; Fig. 5.1). The SSH procedure was performed with the PCR Select cDNA Subtraction kit (BD Biosciences-Clontech) following the manufacturer's recommendations. The PCR mixture containing up-regulated gene fragments (subtracted tester, ST) was cloned using the TOPO TA Cloning kit (pCR 4® vector; Invitrogen, Carlsbad, CA, USA) and transformed into

TOP10 electro competent cells (Invitrogen) using a Gene Pulser II (Biorad, Mississauga, ON, Canada) at a setting of 2.4 kV, 25 μF, and 200 Ohms in Gene pulser cuvettes (Biorad). *E. coli* cells were plated on LB ampicillin (50 μg/mL) plates. A total of 2471 clones were individually collected after 24 h, transferred in 700 μl liquid LB ampicillin to 2-ml tubes for overnight growth at 37 °C. These cultures were used to establish stocks (40% glycerol stored at -80 °C) and to amplify the inserts by PCR.

5.3.4. Microarray differential screening

To confirm differential gene expression, a cDNA microarray was created by printing amplicons derived from each cDNA clone isolated from the SSH library. Briefly, each clone was grown in 200 μl LB-ampicilin O/N at 37 °C and diluted 50-fold in sterile ddH_2O to be used as PCR template. The SSH nested primers (BD Biosciences-Clontech) that flank each insert in the plasmid were used to amplify the inserts and to insure that multiple insert clones were excluded. The 100-μl PCR reactions (2 μl template, 10 μl of 10x PCR buffer (Invitrogen), 200 μmol of each of the dNTPs, 200 nmol of SSH nested primers, 1.5 mmol $MgCl_2$, and 1U Taq polymerase (Invitrogen) were initially incubated at 94 °C for 10 min, then subjected to 30 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 1.5 min, followed by an extension cycle at 72 °C for 10 min. Five μl of each reaction was analyzed by electrophoresis in 1% 1x TAE agarose gels and clones with no insert or producing multiple amplicons were discarded. Single PCR products were purified using a vacuum manifold and Multiscreen FB-plate (Millipore, Nepean, ON, Canada) following the manufacturer's recommendations. Final elution took place in UV-transparent 96-well plates (Corning, Big Flats, NY, USA). Concentrations were evaluated by spectrophotometry using ELx800™ fluorescence reader (Bio-Tek Instruments, Winooski, Vermont, USA). Two μg of purified products were transferred to 96-well V-plates (Corning), lyophilized, resuspended in 50% DMSO and 50% Ultra pure water to give a final concentration of 0.2 $\mu g.\mu l^{-1}$, and arrayed on glass slides with a Virtek printer

(Chipwriter Pro SDD2, Virtek, Ontario, Canada). A total of 2166 amplicons of cDNAs clones were spotted in triplicate on Corning GAPS II slides (Corning).

5.3.5. Hybridization

Two types of hybridization probes were used (*i*) SSH generated probes from the different cDNA libraries: unsubtracted tester (UT), unsubtracted driver (UD) and subtracted tester (ST) for differential screening (Fig. 5.1), and (*ii*) nrDNA ITS (GenBank accession nos. DQ369856, DQ369857), small (GenBank accession nos. DQ369862, DQ369858) and large (GenBank accession nos. DQ369855, DQ369861 and DQ369859, DQ369860) ribosomal subunit genes from *S. elegans* and *R. solani*, to check for nrRNA redundancy. For differential screening, a total of eight hybridizations were carried out including two technical replicates of the ST/UD and the ST/UT hybridizations and their respective dye-swaps. A single hybridization was performed with a mix of nrDNAs (Table 5.1). All probes were prepared by direct labeling with the BioPrime labeling kit (Invitrogen) and Cy3/Cy5 dyes (Amersham Biosciences Piscataway, NJ, USA). The concentration of the incorporated dye was calculated using ND-1000 spectrophotometer.

Pre-hybridization buffer (5x SSC, 0.1% SDS, 0.05% BSA) was sterilized by filtration (0.22 µm) and pre-heated at 42 °C. Twenty µl were deposited on the surface of the slides, covered with cover slips, and incubated in Microarray Hybridization Chambers (Corning) for 1 h at 42 °C. Cover slips were then removed by dipping the arrays in filtered 0.1x SSC, washed twice with 0.1x SSC and finally dried under a clean air stream. Hybridization was carried out in a total volume of 20 µl consisting of at least 1/3 volume of Dig Easy Hyb (Hoffmann-La Roche Ltd, Mississauga, ON, Canada) and dye Cy3- and Cy5-labeled probes (5 pmol from cDNA and 3 pmol from nrDNA). The different types of labeled cDNAs in the hybridization buffer were heated at 95 °C for 5 min and chilled on ice for 1 min. Probes were placed onto the center of the arrays covered with cover slips in sealed hybridization chambers (Corning) and immediately incubated at 42 °C in a water bath for 17 h. Following hybridization, the cover slips were washed

off in 1x SSC, 0.2% SDS preheated to 42 °C, then the slides were transferred in successive 1x SSC, 0.2% SDS baths with gentle shaking (42 °C for 10 min, 37 °C for 5 min, room temperature for 5 min). The slides were then incubated for 5 min at room temperature in 0.1x SSC and dried.

5.3.6. Microarray data analysis

Microarray slides were scanned using the ScanArray Express HT (Perkin-Elmer, Vaudreuil-Dorion, Qc, Canada). Separate images were acquired for separate fluorochromes at a resolution of 10 μm per pixel. Data analysis was performed with ScanArray Express software (Perkin Elmer). After segmentation, local background correction and global normalization using LOWESS (locally weighted polynomial regression; Cleveland, 1979), spots with fluorescence signal intensity < 400 pixels, with a signal-to-noise ratio of < 2, or replicate spots with a standard deviation for the Cy5/Cy3 ratio greater than 2 were discarded. The median intensities of replicate spots were $\log_2$ transformed and analyzed using the following formula using Equation [5.1] developed (van den Berg et al. 2004).

UT/UD = antilog (ER1 – ER2) in base 2 [5.1]

Where :

ER1 = ½ [($\log_2$ Cy3 ST/Cy5 UD) – ($\log_2$ Cy3 UD/Cy5 ST)]

ER2 = ½ [($\log_2$ Cy3 ST/Cy5 UT) – ($\log_2$ Cy3 UT/Cy5 ST)]

ER1 and ER2 are enrichment ratios of ST/UD and ST/UT, respectively, compiled from slides hybridized with ST and UD, and ST and UT, respectively (van den Berg et al., 2004). UT/UD ratio equals the antilog of (ER1-ER2) in base 2 (van den Berg et al., 2004).

5.3.7. Sequence analysis and data handling

Single pass sequencing of 261 cDNA clones was performed at the Genome Quebec Innovation Center (McGill University, Montreal, Qc, Canada) using the M13 universal primers. Sequence analysis was carried out with Chromas 2.3 (http://www.technelysium.com.au/chromas.html) and Biology WorkBench 3.2 (http://workbench.sdsc.edu/). Sequences were then analyzed by BLAST. Stand-

alone BLAST (n and x) analyses were first run against local databases (constructed in mid-September 2005) and comprising all ascomycete and basidiomycete fungal DNA sequences available on NCBI. Standard BLAST (n and x) analyses were also conducted at NCBI (Altschul et al., 1997) on sequences from fungi or without any organism restriction included in the nr database.

Additional searches in other databases were performed using the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST Database version 1.5 (http://cbr-rbc.nrc-cnrc.gc.ca/services/cogeme/), the *Magnaporthe grisea* Genome Database release 2.3 (http://www.broad.mit.edu/annotation/fungi/magnaporthe/index.html), as well as the *Neurospora crassa* Genome Database release 7 (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html). Searches were performed with BLAST (x and -n algorithms) available at the respective web sites using a cut-off E-value of $1e^{-2}$. In addition, search for domains of non-inferred cDNA sequences was done using IntreProScan (http://www.ebi.ac.uk/InterProScan/) and SMART (Letunic et al., 2006; Schultz et al., 1998); http://smart.embl-heidelberg.de/) programs. The redundancy of the 261 cDNA sequences was determined by comparing forward and reverse strands of all sequences with one another using the program ClustalW (http://workbench.sdsc.edu/). Redundant clones with the best BLAST scores are reported in Table 5.2.

5.3.8. Primer design and PCR analysis

To determine the fungal origin of the cDNA sequences, primer pairs were designed for the 120 unique genes using Primer 3 (Rozen and Skaletsky, 2000) and tested in conventional PCR against *S. elegans* and *R. solani* total RNA and gDNA. Primer sets (Table 5.1) flanking 13 selected target genes were further tested in QRT-PCR against the tester and driver cDNA pools in order to validate the up-regulation of the genes.

All conventional PCR reactions were performed in an Applied Biosystems 9600 (Foster City, CA, USA) with primers synthesized by AlphaDNA (Montreal,

QC, Canada). PCR products were resolved on agarose gels (1%, 1x TAE) with Gene Ruler™ 100 bp DNA Ladder (Invitrogen), stained with ethidium bromide and pictures were recorded by a gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON, Canada). Amplification was performed in a 27 µl PCR reactions containing 1x PCR buffer (Invitrogen), 200 µmol dNTPs, 200 µmol primers, 1.5 mmol $MgCl_2$, and 1U Taq polymerase (Invitrogen). The conditions were 10 min at 94 °C (hot start), 35 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, and then an extension cycle at 72 °C for 10 min.

QRT-PCR assays were conducted on 14 transcripts (13 target genes and one housekeeping gene (HKG), histone 4; Table 5.1). One µg of RNA from the tester and the driver was retro-transcribed into cDNA using RT Quantitech kit (QIAGEN) and the tester's and driver's cDNAs were diluted to $1/20^{th}$. Three technical replicates were performed for each of the two templates and a negative control was included with each replicate in each run. QRT-PCR was conducted in Mx3000 (Stratagene, Cedar Creek, USA) and SYBR Green master mix (Stratagene) following the manufacturer's recommendations. Amplification was performed in a 24 µl reaction mixture containing 125 nmol of each primer, 1x SYBR Green master mix, 30 nmol of reference dye ROX, and 2 µl of cDNA template. The amplification conditions were 95 °C for 10 min (hot start), followed by different number of cycles and annealing temperatures (Table 5.1) and then an extension at 72°C for 10s. The fluorescence reading was done at 72°C at the end of the elongation cycles, except for clone 1144 which was held at 80°C for 11 s. Following amplification, a melting curve was generated by programming the thermocycler to reach 95 °C (60 s), 55 °C (30 s) (2.5 $°C.s^{-1}$) and 95 °C (0 s) (0.1 $°C.s^{-1}$). Data generated by QRT-PCR were estimated using Stratagene analysis software.

The relative expression ratios of the 13 target genes in the tester cDNA pool (sample) versus the driver (control) were calculated using the procedure described earlier (Pfaffl, 2001). The PCR amplification efficiencies were calculated from equation [5.2] (Pfaffl, 2002; unpublished data; http://www.gene-quantification.info/). RnA and RnB are reporter fluorescence at cycle n at

arbitrary thresholds A and B, CPA and CPB are the threshold cycles at these arbitrary thresholds.

$$E = (RnB/RnA)^{[1/CPB - CPA]} \qquad \text{Equation [5.2]}$$

5.4. RESULTS

5.4.1. Creation of a subtracted cDNA library

An SSH library was constructed with RNA isolated at several sequential time points during the mycoparasitic interaction between *S. elegans* and its host *R. solani*. These time points were chosen to cover all stages of mycoparasitism and investigate expressed genes both before and during the interaction. In order to restrict the analysis to the mycoparasitic process and defense reaction of the host, subtraction was performed between the mycoparasitic interaction cDNAs (tester) and the cDNAs of each of the interacting partners alone (driver) (Fig. 5.1). Gene specific primers (Table 5.1) were used to monitor the pre- and post-SSH enrichment levels of the housekeeping gene histone-4 targeting *S. elegans* only. There was a significant reduction in the abundance of histone-4 in the enriched library, indicating that the subtraction method was successful (data not shown).

A total of 2166 amplicons, amplified from clones containing single inserts that ranged from 200 to 1,300 bp, were spotted in triplicate on microarray glass slides and screened with combinations of SSH generated probes. Hybridization with nuclear ribosomal DNA (nrDNA) as probes showed that only 52 (2.4%) out of 2166 represent nrDNA.

5.4.2. Differential screening

We have used the microarray analysis to identify false positives instead of gene expression, in a similar way as dot blot analyses or macroarray analyses. Thus, biological replicates were not required. Enrichment ratios (ER1 and ER2) were calculated for each amplicon on the array and plotted against each other (Fig. 5.2). Positive ER1 and ER2 values indicate transcript enrichment during SSH relative to their levels in UD or UT respectively (Fig. 5.1B). Compilation of

the microarray data generated ER1, ER2 values, and UT/UD ratios (Fig. 5.2, Table 5.2) confirmed that 1016 genes out of the 2166 (47%) were up-regulated in the tester compared to the driver (ER1>ER2), while the rest of the sequences (1150, 53%) had escaped subtraction (ER1<ER2), being as abundant in the driver as in the tester. Among the up-regulated candidates, a total of 429 sequences had negative ER2 values suggesting they correspond to abundant transcripts, while 587 sequences had positive ER2 values suggesting they correspond to rare transcripts that had been enriched in the SSH library (Fig. 5.2).

5.4.3. Sequence analysis and annotation of expressed sequence tags (ESTs)

Out of the 1016 genes showing differential expression, 253 showing a UT/UD ratio higher than 1.20, and eight additional amplicons having a UT/UD ratio between 0.97 and 1.20 were randomly picked and sequenced giving a total of 261 sequences. The sequences were analyzed using the program ClustalW in order to identify redundant clones. A total of 120 unique ESTs were identified of which 95 appeared only once and 25 were represented by multiple sequences at frequencies ranging from 2 to 47 (Fig. 5.3) with EST 1144 having the highest occurrence (Table 5.2). The sequences of the *S. elegans/R. solani* clones were submitted to NCBI as GenBank accession nos. DQ369798-DQ369846, DQ369848, DQ369849, DQ369855-DQ369862, DW520683-DW520893, DW714026 (Appendix I).

PCR analysis using designed specific primers showed that out of 120 unique sequences, 77.5% (93 ESTs) belong to the mycoparasite *S. elegans*, 12.5% (15 ESTs) belong to *R. solani*, 1.7% (2 ESTs) were amplified in both organisms, while 8.3% (10 ESTs) were not amplified (Figure 5.4A). Putative functions were assigned for 39 sequences (33.5%) (Fig. 5.4B; Table 5.2). Another 11 sequences (9.2%) showed high homology with mitochondrial sequences, while 17 EST sequences (14.2%) had high similarity scores in the databases but not associated with known function and were considered unclassified (Fig. 5.4B; Table 5.2). A total of 43 sequences (35.8%) had no match with sequences in the public databases (Table 5.2, Fig. 5.4B), and thus were considered as novel sequences.

Only 8.3% of the sequence clones (10) had multiple but possibly distinct functional roles. This functional multiplicity were kept and presented as such in Table 5.2. Of interest, ESTs 151 and 1144 showed high homology with different genes related to biosynthesis of toxins or pathogenesis. Both are similar to each other and to ESTs 3043 and 2404 which had no match.

The 39 putative genes for which functions were identified were categorized into functional classes (Table 5.2, Fig. 5.4C). Genes related to pathogenic processes formed the largest category corresponding to 23% of total unique sequences, followed by associate with toxin metabolism (Fig. 5.4C). The genes involved in vitamin metabolism were identified as pyridoxal reductase (Table 5.2), while the genes involved in toxin metabolism, included a hexose transporter (EST 748), a cytochrome P450 similar to trichothecene C-15 hydrolase (EST 2557), an O-methyltransferase B (EST 1729), an acetylcholinesterase (EST 3340), and a transcription factor $Zn(II)_2Cys_6$ (EST 4522). We also identified nine genes which are likely to be involved in pathogenic processes, two of which encode short chain dehydrogenase/reductases. The remaining genes were identified as a copper transporter, a ferric-chelate reductase, a calmodulin, and an ankyrin repeat protein associated with an F-box domain. Other genes closely related to pathogenesis were grouped into three different classes, stress response, multidrug resistance, and apoptosis (Table 5.2; Fig. 5.5C). Genes encoding transport-related proteins (Table 5.2) represented 7.7% of the sequenced ESTs, including the proteins *Yop* (EST 2065) and *Mog1* (EST 5977). The other inferred genes were grouped into classes related to more general metabolic processes (Fig. 5.4C). The class designated as others included genes (e.g. the ribosomal protein L10), whose possible role during the pathogenic interaction is not clear. (Table 5.2, Fig. 5.4C).

To gain more information on the identity of the assigned genes, translated protein domain searches were conducted using the programs InterProScan and SMART. Several genes presented a signal peptide and transmembrane regions (Table 5.2). Domains have been assigned to six putative genes, ESTs 514, 1042, 2932, 2950, 5200, and 6358 among which three were unclassified (5200, 1042,

and 2950), one as mixed (6358), one as mitochondrial (514), and one as no match (2932). EST 6358 has an alpha/beta hydrolase domain and belongs to the carboxylesterase type B family. This information suggested that it is an acetylcholinesterase. EST 2932 contains a NATCH domain which is retrieved in several protein families. EST 5200 contains a WD-40 repeat domain which is involved in protein-protein interactions. EST 1042 appears to be a member of the ferredoxin reductase family as it has an oxidoreductase NAD-binding domain similar to that found in ferric-chelate reductases. EST 514 has a tonB domain with receptor protein signature, while EST 2950 contains a Von Willebrand factor type A (VWA) domain that is a multimeric glycoprotein involved in adhesion via metal independent adhesion sites in human.

5.4.4. Validation and QRT-PCR expression analysis of selected clones

To validate conclusions drawn from ER1 and ER2 comparisons and to confirm that the isolated sequences are preferentially expressed transcripts, QRT-PCR was performed on tester and driver cDNA pools for 13 selected genes belonging to *S. elegans* and assigned to various functions (Table 5.2). All of the QRT-PCR ratios corroborated well with UT/UD ratios (Table 5.3) (i.e., clones having UT/UD >1 gave QRT-PCR ratios >1) confirming up-regulation of the genes during the mycoparasitic interaction. Four of the selected genes (ESTs 1546, 2968, 151, and 1144), identified as having multiple functions or no match, were highly overexpressed during the mycoparasitic interaction (Table 5.3).

Table 5.1. PCR Primer characteristics.

Target	Clone ID	QRT-PCR conditions	Primer	Sequence (5'- 3')	Tm (°C)[a]	Amplicon size (bp)
Internal Transcribed Spacers (ITS) 1 and 2			ITS4[b]	TCCTCCGCTTATTGATATGC	58	Se ~ 600[f]
			ITS5[b]	GGAAGTAAAAGTCGTAACAAGG	63	Rs ~ 750[f]
Fungal ITS rRNA small subunit			NS1[b]	GTAGTCATATGCTTGTCTC	54	~1800
			NS8[b]	TCCGCAGGTTCACCTACGGA	64	
Fungal nrRNA large sub-unit part 1			LR0R[c]	ACCCGCTGAACTTAAGC	52	Se ~ 1300[f]
			LR7[c]	TACTACCACCAAGATCT	48	Rs ~ 1900[f]
Fungal nrRNA large subunit part 2			LR7R[c]	GCAGATCTTGGTGGTAG	52	Se ~ 1700[f]
			LR12[c]	GACTTAGAGGCGTTCAG	52	Rs ~ 2000[f]
Ankyrin repeat protein	1867	B	Ankyrin-F	TACTCTCAACACTCAGGACCGCTT	72	138
			Ankyrin-R	TCACATGGACTCATCGTTGTCGCAT	72	
Mixed functions	1546	C	1546-F	GAGAACAGCAGTCTTCATTTC	62	111
			1546-R	CTAAGTATAGCACCAGAGGCA	60	
Transcription factor	4522	A	TF-F	CGACCTTGTATAGCGTGCGAAGTT	72	118
			TF-R	TTCTACAATGCTAGGCCCTTTGCG	72	
No match	4801	D	4801-F	CCCTCTTGTTGCCCTTTCCTTTGT	72	140
			4801-R	ACGTTGGTGAGACCTAGCATCGAG	74	
Yop	2065	B	Yop-F	TGACTTACTGGGTCGTCTTTGCCT	72	111
			Yop-R	AGAGCCACAGCAGGAAGATGAACT	72	
Mog	5977	A	Mog-F	TCGATCAGGATGGTTTCACCAGCA	72	129
			Mog-R	TGATATCGGTGCCAACCATGTCCT	72	
MFS hexose transporter	5641	A	MSFHT-F	CCAGATTGCCTTTGTCTGGCTGTT	72	119
			MSFHT-R	TGATCATCATACCCTTGGCACGGA	72	
Cytochrome P450	2557	B	Cytc-F	AGATGCGAGTGGCGCAAGTTCTTT	72	137
			Cytc-R	TTCGCAGCGACTCGAGAACCATTA	72	
Calmodulin	4645	A	Calmod-F	CGGCAGAGATGAAACCGTTGTTGT	72	120
			Calmod-R	TTGACTTCCCAGAGTTCCTGACCA	72	
Ribosomal protein L10	6535	B	L10-F	TATCCCATGTCGCGATTCAACCGT	72	133
			L10-R	TCAGCTGCTCATACTCGTTGGAGA	72	

Table 5.1. PCR Primer characteristics. (Continued)

Target	Clone ID	QRT-PCR conditions	Primer	Sequence (5'- 3')	Tm (°C)[a]	Amplicon size (bp)
			L10-R	TCAGCTGCTCATACTCGTTGGAGA	72	
Mixed functions	151	D	151-F	AATACGCCATACAGCCGAGAGACA	72	110
			151-R	TCAAGACGCCCGATTGGTTCTGAT	72	
No match	2968	B	2968-F	ACGCTGTCACGCATATACCAATCC	72	134
			2968-R	TCTCGGGCCATCCTTGGAATACTT	72	
Mixed functions	1144	E	655-F	TCTACACCTTGGACTCCATCTC	66	416
			655-R	GAGCCTCGAGTGTTTCTGATTC	66	
Histone 4[d]			H4-1a[d]	GCTATCCGCCGTCTCGCT	60	200
			H4-1b[d]	GGTACGGCCCTGGCGCTT	62	
Histone 3[d]			H3-1a[d]	ACTAAGCAGACCGCCCGCAGG	70	390
			H3-1b[d]	GCGGGCGAGCTGGATGTCCTT	70	
ß-tubulin			SBU177[e]	TTTGGATGTGGGGTCTTTGC	60	179
			SBL336[e]	AACATAGTGCCTTCTCTTCA	56	

a Tm is provided as Tm = 2x(A+T)+4x (C+G))
b White et al., 1990
c Vilgalys' lab, http://www.biology.duke.edu/fungi/mycolab/primers.htm
d Glass and Donaldson, 1995
e Thon and Royse, 1999
f SE = *S. elegans*; RS = *R. solani*

A = 40 cycles at 95 °C for 30 s, annealing at 61 °C for 1 min.
B = 53 cycles at 95 °C for 30 s, annealing at 64 °C for 1 min.
C = 50 cycles at 95 °C for 30 s, annealing at 58 °C for 1 min.
D = 40 cycles at 95 °C for 30 s, annealing at 64 °C for 1 min.
E = 45 cycles at 95 °C for 30 s, annealing at 61 °C for 1 min.

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver.

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
FUNCTION INFERRED										
Toxin and toxin metabolism										
DW520693	3340	Acetylcholinesterase	*Bungarus fasciatus*	1E-05	1		0,28	0,68	1,32	*S. elegans*
DW520689	2557	Cytochrome P450	*Aspergillus fumigatus*	2E-21	1		-0,13	0,17	1,23	*S. elegans*
		trichothecene C-15 hydroxylase	*Fusarium sporotrichioides*	2E-10						
DW520736	748	MFS hexose transporter	*Aspergillus fumigatus*	2E-84	4		0,50	1,10	1,51	*S. elegans*
DW520859	1729	O-methyltransferase B	*Neurospora crassa*	1E-08	1		-0,01	0,29	1,23	*S. elegans*
DW520684	4522	Transcription factor Zn(II)2Cys6	*Emericella nidulans*	1E-06	1		1,82	2,22	1,32	*S. elegans*
Pathogenic processes										
DW520865	667	Short-chain dehydrogenase/reductase	*Cryphonectria parasitica*	2E-09	7		0,42	0,76	1,27	*S. elegans*
DW520868	4990	Short-chain dehydrogenase/reductase	*Cryphonectria parasitica*	2E-09	1		2,27	2,85	1,50	*S. elegans*
DW520713	2089	Ctr copper transporter family	*Aspergillus fumigatus*	2E-09	1		0,30	0,76	1,37	*S. elegans*
DW520719	2791	Ferric-chelate reductase	*Aspergillus fumigatus*	1E-19	1		-0,19	0,21	1,32	*S. elegans*
DW520720	5053	Ferric-chelate reductase	*Cryptococcus neoformans*	6E-04	1		-0,12	0,25	1,29	ND
DW520690	4645	Calmodulin	*Neurospora crassa*	2E-156	2		-0,27	0,01	1,21	*S. elegans*
DW520712	6802	Calpain-like protease	*Neurospora crassa*	4E-26	1		0,38	0,72	1,27	*S. elegans*
DW520683	1867	Ankyrin repeat protein	*Aspergillus fumigatus*	1E-03	1		2,73	3,36	1,55	*S. elegans*
		Zinc finger, ZZ type domain protein	*Aspergillus fumigatus*	3E-03						
		F-box domain	*Aspergillus fumigatus*	7E-03						
DW520694	1894	Alcohol oxidase	*Cochliobolus victoriae*	1E-10	1		-0,21	0,12	1,26	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
Stress response										
DW520723	3439	Glycogen phosphorylase	*Aspergillus fumigatus*	2E-29	1		-0,21	0,09	1,24	*S. elegans*
Multidrug resistance										
DQ369839	2044	40S ribosomal protein S13	*Mycosphaerella graminicola*	5E-03	1		0,06	0,43	1,30	*S. elegans*
Apoptosis										
DW520735	4348	Importin-alpha export receptor	*Neurospora crassa*	2E-55	1		-0,21	0,08	1,22	*S. elegans*
DW520861	3760	Proteasome subunit alpha type 3	*Cryptococcus neoformans*	5E-50	1		-0,30	0,19	1,41	*R. solani*
Transport										
DW520862	2959	Purine permease	*Aspergillus nidulans*	3E-42	1		0,02	0,29	1,21	*R. solani*
DW520685	2065	Protein Yop-1	*Neurospora crassa*	1E-62	1		-0,83	-0,35	1,39	*S. elegans*
DW520686	5977	Ran guanine nucleotide release factor Mog1p	*Candida albicans*	7E-13	2		1,62	2,46	1,79	*S. elegans*
Vitamin metabolism										
DW520698	5146	Pyridoxal reductase (AKR8)	*Aspergillus fumigatus*	6E-25	9		2,85	3,32	1,39	*R. solani*
DW520864	3391	Pyridoxal reductase (AKR8)	*Aspergillus fumigatus*	4E-25	8		2,23	2,71	1,39	*R. solani*
ATP synthesis										
DW520739	937	NADH-ubiquinone oxidoreductase chain 3	*Hypocrea jecorina*	3E-10	1		0,02	0,30	1,21	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
Respiration chain										
DW520714	2305	Cytochrome c	*Ustilago maydis*	4E-44	1		-0,14	0,19	1,26	*R. solani*
DW520715	5500	Cytochrome c oxidase subunit 3	*Epidermophton floccosum*	1E-22	1		0,06	0,38	1,24	*R. solani*
DQ369804	4552	40S ribosomal protein S9	*Ustilago maydis*	1E-83	2		0,03	0,32	1,22	*R. solani*
Replication, transcription, and DNA repair										
DW520716	3046	DNA ligase	*Aspergillus fumigatus*	6E-47	1		2,51	2,26	1,19	*S. elegans*
DW520724	5257	Histone H2A	*Neurospora crassa*	1E-123	1		-0,66	-0,31	1,27	*S. elegans*
Translation										
DQ369842	2599	60S ribosomal protein L12	*Hypocrea jecorina*	9E-90	1		-0,36	-0,04	1,24	*S. elegans*
Transduction										
DW520710	6889	Ankyrin repeat protein	*Rattus norvegicus*	3E-04	1		-0,07	0,24	1,24	ND
Protein degradation										
DW520873	2380	Ubiquitin 1	*Gibberella pulicaris*	1E-126	2		-0,16	0,25	1,33	both
Ribosomal protein										
DQ369823	2029	Ribosomal protein S28	*Hypocrea jecorina*	3E-125	1		-0,24	0,19	1,35	*S. elegans*
DQ369820	4465	Ribosomal protein S28	*Hypocrea jecorina*	4E-136	1		-0,39	-0,01	1,30	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
Others										
DW520722	2155	Glycine cleavage system H protein	*Sinorhizobium meliloti*	6E-03	1		1,45	2,06	1,53	*S. elegans*
DW520717	5830	Dynamin GTPase	*Aspergillus fumigatus*	3E-94	1		-0,51	-0,20	1,24	*S. elegans*
DQ369806	6535	Ribosomal protein L10	*Hypocrea jecorina*	9E-99	2		0,25	0,53	1,21	*S. elegans*
DW520718	5899	Endoplasmic reticulum protein	*Cryptococcus neoformans*	4E-07	1		0,58	1,19	1,52	*R. solani*
DW520721	2674	Glucose-6-phosphate 1-dehydrogenase	*Aspergillus niger*	9E-63	1		-0,17	0,10	1,21	*S. elegans*
NON INFERRED										
Mitochondrial RNA										
DQ369848	2122	Mitochodrial DNA	*Fusarium oxysporum*	8E-07	1	SP-TM	2,19	2,66	1,38	*S. elegans*
DQ369812	2275	Mitochodrial DNA	*Hypocrea jecorina*	9E-25	10	SP	-0,57	-0,19	1,30	*S. elegans*
DQ369809	2281	Mitochodrial DNA	*Hypocrea jecorina*	3E-66	5	SP	-1,42	-0,90	1,43	*S. elegans*
DQ369834	2704	Mitochodrial DNA	*Fusarium oxysporum*	2E-154	2	SP-TM	-0,47	-0,16	1,24	*S. elegans*
DQ369828	3457	Large subunit ribosomal RNA	*Hypocrea citrina*	1E-56	2	SP	-0,83	-0,39	1,36	*S. elegans*
DQ369799	1720	Large subunit ribosomal RNA	*Neurospora crassa*	7E-95	1	SP-TM	3,04	4,05	2,02	*S. elegans*
DQ369815	514	Small subunit ribosomal RNA	*Cordyceps capitata*	5E-96	15	1	-0,65	-0,11	1,45	*S. elegans*
DQ369814	1318	Small subunit ribosomal RNA	*Botryoshaeria dothidea*	8E-16	1	SP	-0,76	-0,47	1,23	*S. elegans*
DQ369821	1345	Small subunit ribosomal RNA	*Cordyceps ramosopulvinata*	3E-83	1	SP	-0,44	0,06	1,41	*S. elegans*
DQ369813	3085	Small subunit ribosomal RNA	*Hypocrea pseudokoningii*	1E-75	2		-1,38	-1,07	1,23	*S. elegans*
DQ369846	3937	Small subunit ribosomal RNA	*Verticillium lipolytica*	2E-32	1	SP-TM	-1,16	-0,80	1,29	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
Unclassified										
DQ369801	3382	Related to ribosomal protein MRP49	*Neurospora crassa*	3E-20	1		-0,08	0,28	1,28	*S. elegans*
DW520725	2950	Hypothetical protein	*Gibberella zeae*	1E-06	1	2	-0,19	0,15	1,26	*S. elegans*
DW520726	3298	Hypothetical protein	*Aspergillus fumigatus*	9E-16	1	SP-TM	0,55	0,83	1,22	ND
DW520727	4897	Hypothetical protein	*Magnaporthe grisea*	9E-21	1	SP-TM	1,17	1,48	1,25	*S. elegans*
DW520728	4927	Hypothetical protein	*Aspergillus nidulans*	7E-10	1	SP-TM	0,39	0,90	1,42	*S. elegans*
DW520729	5200	Hypothetical protein	*Neurospora crassa*	2E-12	1	3	0,34	0,61	1,20	*S. elegans*
DW520730	5578	Hypothetical protein	*Ustilago maydis*	2E-27	1		2,38	3,14	1,69	*R. solani*
		Similar to prostatic acid phosphatase rPAP	*Phanerochaete chrysosporium*	3E-05						
DW520731	604	Hypothetical protein	*Cryptococcus neoformans*	3E-15	1		-0,14	0,15	1,23	*R. solani*
DW520732	1042	Hypothetical protein	*Gibberella zeae*	5E-13	1	4-5	-0,21	0,05	1,20	*S. elegans*
DW520733	1264	Hypothetical protein	*Gibberella zeae*	3E-05	1	TM	0,36	0,69	1,26	*S. elegans*
DW520860	853	Predicted protein	*Neurospora crassa*	4E-12	1		-0,01	0,28	1,22	*S. elegans*
DW520875	6727	Unknown	*Cryphonectria parasitica*	8E-03	1		0,14	0,68	1,46	*S. elegans*
DW520882	2023	Unknown	*Gibberella zeae*	8E-77	2	SP	0,87	1,20	1,26	*S. elegans*
DW520884	2659	Unknown	*Gibberella zeae*	2E-15	1		-0,75	-0,14	1,52	ND
DW520888	3970	Unknown	*Platystomus albinus*	5E-06	1		2,82	3,17	1,28	*S. elegans*
DW520889	4111	Unknown	*Gibberella zeae*	7E-03	1	SP	-0,63	-0,36	1,20	*S. elegans*
DW520892	6097	Unknown	*Cryphonectria parasitica*	7E-03	1		0,39	0,73	1,27	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
Different significant similarities (mixed functions)										
DW520691	151	Geranylgeranyl diphosphate synthase	*Fusarium proliferatum*	1E-36	1		1,43	2,31	1,84	*S. elegans*
		Ammodytin	*Vipera ammodytes*	8E-36						
		Metallopanstimulin	*Strongloides ratti*	3E-32						
		Integron IntI	*Citrobacter freundii*	1E-31						
DW520876	1813	Unknown	*Hypocrea jecorina*	4E-05	1		3,05	3,76	1,63	ND
		Glutamine synthase	*Paxillus involutus*	7E-04						
		Translation release factor erf3	*Paxillus involutus*	7E-04						
		Alpha-crystallin related protein	*Trichophyton mentagrophytes*	7E-04						
DW520878	766	Unknown	*Hypocrea jecorina*	3E-07	1	SP-TM	2,78	3,41	1,55	*S. elegans*
		Histone H4.1	*Hypocrea jecorina*	4E-06						
		Tripeptidyl peptidase I	*Paxillus involutus*	2E-05						
		Glyceraldhyde 3-phosphate dehydrogenase (GAPDH)	*Hypocrea jecorina*	2E-05						
DW520879	1144	Unknown	*Cronartium quercuum*	4E-16	47		2,43	2,18	1,19	*S. elegans*
		Metallopanstimulin	*Strongloides ratti*	4E-11						
		CipC protein	*Paxillus involutus*	2E-08						
		Ammodytin	*Vipera ammodytes*	6E-07						
DW520881	1546	Unknown	*Hypocrea jecorina*	4E-07	1		2,71	3,41	1,63	*S. elegans*
		Alcohol dehydrogenase	*Arachis hypogaea*	7E-05						
		Glutathione S-transferase	*Ixodes ricinus*	2E-04						
		Hydrophobin-3 precursor	*Paxillus involutus*	1E-04						

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
DW520885	2707	Unknown	*Metarhizium anisopliae*	0E+00	1	SP-TM	-0,26	0,36	1,54	both
		Cytochrome P450 monooxygenase	*Magnaporthe grisea*	0E+00						
		Unspecific monooxygenase	*Hypocrea jecorina*	0E+00						
		25S ribosomal RNA	*Trichoderma reesei*	0E+00						
DW520886	2962	Unknown	*Paxillus involutus*	3E-09	1	SP	-0,20	0,26	1,37	*S. elegans*
		Hydrophobin-3 precursor	*Paxillus involutus*	5E-08						
		Proteasome 26S subunit	*Paxillus involutus*	5E-08						
		CipC protein	*Paxillus involutus*	5E-08						
DW520887	3775	Unknown	*Gibberella moniliformis*	9E-80	1	SP	-2,54	-0,61	3,81	*S. elegans*
		Mitochondrial DNA	*Fusarium oxysporum*	2E-10						
DW520891	5329	Unknown	*Alternaria brassicicola*	1E-08	1	SP	2,57	3,17	1,52	*S. elegans*
		CipC protein	*Paxillus involutus*	6E-04						
		Glutathione S-transferase	*Ixodes ricinus*	3E-04						
		Metallopanstimulin	*Strongloides ratti*	4E-03						
DW520893	6358	Unknown	*Magnaporthe grisea*	3E-31	1	SP-6-7	-0,50	-0,18	1,25	*S. elegans*
		Acetylcholinesterase	*Bungarus fasciatus*	1E-05						
No significant homology (no match)										
DW520745	313	No match			2		-0,21	0,10	1,24	*S. elegans*
DW520746	379	No match			1		3,43	4,07	1,56	*S. elegans*
DW520750	673	No match			3	SP-TM	2,72	3,39	1,59	*S. elegans*
DW520754	850	No match			20		2,80	3,21	1,34	*S. elegans*
DW520756	970	No match			1	SP	0,20	0,50	1,24	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
DW520759	1072	No match			2		2,44	2,87	1,34	*S. elegans*
DW520769	1651	No match			2	TM	2,48	2,78	1,23	*S. elegans*
DW520774	1957	No match			1		2,27	2,91	1,56	*S. elegans*
DW520776	2062	No match			1	SP	-0,19	0,18	1,29	*S. elegans*
DW520777	2101	No match			1	SP-TM	2,57	3,02	1,37	*S. elegans*
DW520778	2119	No match			2	SP-TM	-0,12	0,29	1,32	*S. elegans*
DW520783	2299	No match			1	SP-TM	3,01	4,53	2,88	*S. elegans*
DW520785	2404	No match			1		2,85	3,47	1,54	*S. elegans*
DW520786	2497	No match			1		-1,33	-1,04	1,22	*R. solani*
DW520790	2680	No match			1	SP	-0,22	0,07	1,22	*S. elegans*
DW520793	2719	No match			1	SP-TM	-0,21	0,30	1,43	*S. elegans*
DW520795	2725	No match			1	SP-TM	-1,00	-0,40	1,52	*R. solani*
DW520796	2806	No match			1	SP-TM	1,13	1,62	1,41	*S. elegans*
DW520797	2899	No match			1	SP-TM	-0,11	0,16	1,20	*S. elegans*
DW520799	2929	No match			1	SP	0,02	0,39	1,29	*S. elegans*
DW520800	2932	No match			1	8-9	-1,14	-0,74	1,33	*S. elegans*
DW520687	2968	No match			10		1,82	2,16	1,27	*S. elegans*
DW520801	3043	No match			2		2,58	2,96	1,31	*S. elegans*
DW520804	3199	No match			1		-0,12	0,23	1,28	ND
DW520805	3247	No match			1	SP-TM	-0,02	0,25	1,21	*S. elegans*
DW520811	3748	No match			1	SP-TM	-0,29	-0,02	1,20	*S. elegans*
DW520812	3832	No match			1		-0,09	0,20	1,22	*S. elegans*

Table 5.2. Differentially expressed cDNAs detected in the microarray differential screening comparing subtracted tester, unsubtracted tester, and unsubtracted driver. (continued)

Accession number$	EST ID	BLAST annotation	Hit organism	E-value	No. of clones	Domain*	ER1	ER2	UT/UD ratio	Organism
DW520813	3838	No match			1	SP	-0,28	0,02	1,23	*S. elegans*
DW520814	3865	No match			1	SP	-2,26	-1,97	1,22	*S. elegans*
DW520820	4183	No match			1		2,78	3,46	1,60	*R. solani*
DW520822	4381	No match			1	SP	0,00	0,35	1,28	*S. elegans*
DW520827	4765	No match			1	TM	0,14	0,43	1,23	*R. solani*
DW520692	4801	No match			1	SP	-0,52	-0,25	1,21	ND
DW520828	4816	No match			1	SP	20,24	23,26	8,11	*S. elegans*
DW520832	5131	No match			1		-0,41	-0,03	1,30	ND
DW520835	5446	No match			1		2,48	2,85	1,29	*R. solani*
DW520837	5491	No match			1	SP	-0,41	-0,13	1,21	ND
DW520839	5560	No match			1	SP	0,13	0,55	1,34	*S. elegans*
DW520840	5590	No match			1		0,32	0,81	1,41	*S. elegans*
DW520844	5737	No match			1	SP	-0,30	0,00	1,23	ND
DW520851	6670	No match			1	SP-TM	0,01	0,38	1,30	*S. elegans*
DW520855	6823	No match			1		2,55	2,83	1,21	*S. elegans*
DW520857	6859	No match			1	SP-TM	-0,52	-0,07	1,37	*S. elegans*

$List of accession numbers, see Appendix I.

*Domain includes domain, family, signal peptide, transmembrane regions. This specification is only for non inferred ESTs.

SP- Signal peptide

TM- Transmembrane regions

1- TonB-dependent receptor proteins signature 1.

2- Von Willebrand factor type A (VWA)

3- WD-40 repeat

4- Oxidoreductase NAD-binding domain

5- Ferredoxin reductase-like family

6- Carboxylesterase, type B family

7- α/β hydrolase

8- NATCH domain

9- P-loop containing nucleoside triphosphate hydrolases

Table 5.3. Expression ratio of 13 randomly selected ESTs.

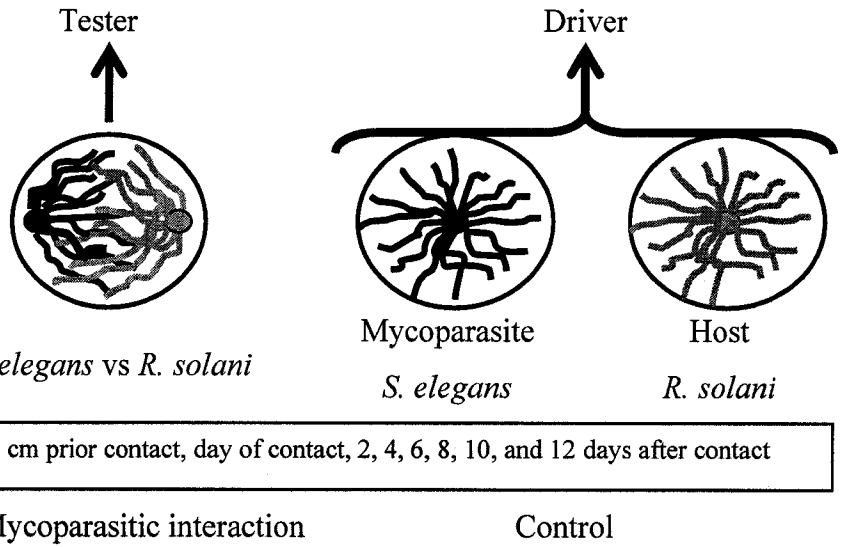
GENE NAME	EST ID	UT/UD*	QRT-PCR RATIO$
Ankyrin repeat protein	1867	1.55	1.11
Mixed functions	1546	1.63	57.33
Transcription factor	4522	1.32	1.12
No match	4801	1.30	1.28
Yop	2065	1.39	1.30
Mog	5977	1.80	1.21
MSF hexose transporter	5641	1.24	1.41
Cytochrome P450	2557	1.23	1.19
Calmodulin	4645	1.21	1.28
Ribosomal protein L10	6535	1.21	1.04
Mixed functions	151	1.84	4.00
No match	2968	1.82	17.65
Mixed functions	1144	1.19	15.08

* Ratios are calculated based on the formulae of van den Berg et al. (2004).

$ Values were normalized against a HKG histone-4 and the relative expression ratios were calculated based on formulae developed by Pfaffl (2001).

Figure 5.1. Scheme for the isolation of RNA from mycoparasitic interaction between the mycoparasite *S. elegans* and its host *R. solani* (tester), and from control condition where both fungi grew in different petri plates (driver). A, RNAs were used for the construction of a suppression subtractive hybridization (SSH) cDNA library to isolate ESTs unique to mycoparasitic interaction (tester). B, designation of SSH cDNA populations used in the differential screening analyses.

A



B

SSH denominations		cDNA content	Organisms
ST	Subtracted tester	cDNAs unique to the tester	*S. elegans* and *R. solani* in interaction during mycoparasitism
UT	Unsubtracted tester	All the tester cDNAs	*S. elegans* and *R. solani* in interaction during mycoparasitism
UD	Unsubtracted driver	All the driver cDNAs	*S. elegans* and *R. solani* not in interaction, growing on separate petri dishes

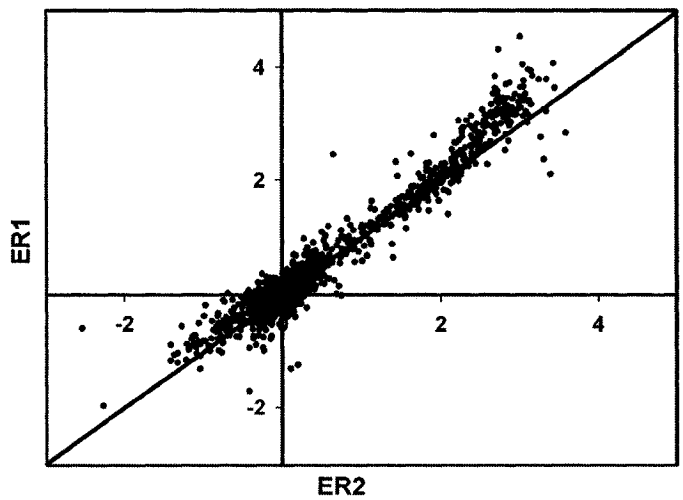


Figure 5.2. Differential screening of *S. elegans-R. solani* SSH library by cDNA microarrays. Suppression subtractive hybridization (SSH) scatterplot showing enrichment ratio 1 (ER1) and enrichment ratio 2 (ER2) for each of the clones. ER1 and ER2 were calculated by log2 transformation of the subtracted tester (ST) median fluorescence divided by the unsubtracted driver (UD) median fluorescence and log2 transformation of the subtracted tester (ST) median fluorescence divided by the unsubtracted tester (UT) median fluorescence, respectively. Clone sequences lying above the diagonal line correpond to truly up-regulated genes (ER1>ER2) while clone sequences lying below the line escaped subtraction. Positive ER2 values indicate that genes had a low expression and have therefore been enriched in the library. Negative ER2 values indicate that genes had relatively high expression and that their abundances had been normalized in the library (van den Berg et al., 2004).

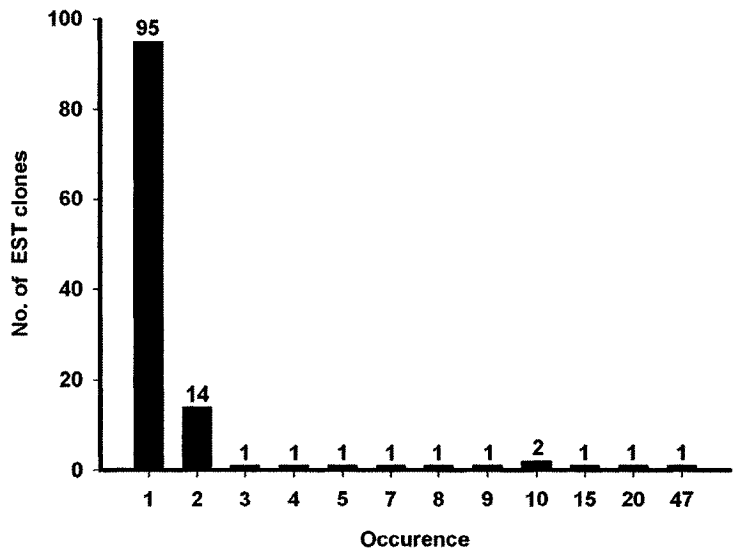
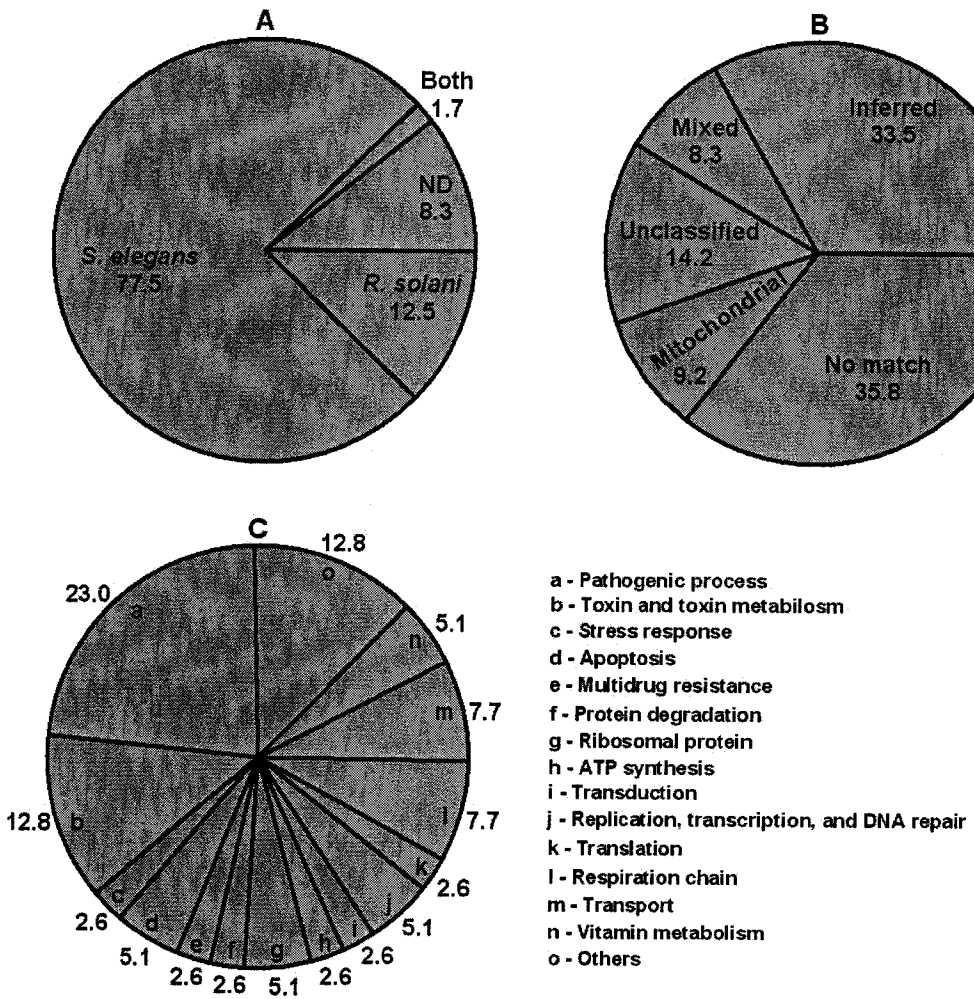


Figure 5.3. The frequency of occurrence of ESTs derived from *S. elegans-R. solani* interaction. The number of ESTs is shown above each of the number of occurrences.

Figure 5.4. Functional distribution of *S.elegans* and *R. solani* up-regulated genes. A, 120 unique sequences were categorized toward their origin and **B**, their putative function. **C**, Putative functional classes for the 39 sequences for which putative function could be inferred. Percentages were calculated from the total 120 up-regulated unique sequences (A and B) and from the 39 sequences with inferred functions (C). Note: "no match" represents sequences that are not similar to any protein in the database, while "unclassified" represents sequences whose matching genes do not have a clear function. ND, not determined, the designed primers failed to amplify these cDNAs.


A
Both
1.7
ND
8.3
S. elegans
77.5
R. solani
12.5
B
Inferred
33.5
Mixed
8.3
Unclassified
14.2
Mitochondrial
9.2
No match
35.8
C
12.8
o
5.1
n
7.7
m
7.7
l
2.6
k
5.1
j
2.6
i
2.6
h
5.1
g
2.6
f
2.6
e
5.1
d
2.6
c
12.8
b
23.0
a
a - Pathogenic process
b - Toxin and toxin metabilosm
c - Stress response
d - Apoptosis
e - Multidrug resistance
f - Protein degradation
g - Ribosomal protein
h - ATP synthesis
i - Transduction
j - Replication, transcription, and DNA repair
k - Translation
l - Respiration chain
m - Transport
n - Vitamin metabolism
o - Others

5.5. DISCUSSION

The EST analysis reported in this study provides an efficient means of gene discovery, particularly for the mycoparasite *S. elegans* for which there have been no molecular and genetic studies. Consistent with the findings of other fungal EST studies (Keon et al., 2000; Liu and Yang, 2005; Qutob et al., 2000), in the current study, nearly 43% of the translated of cDNA sequences showed no similarity to known protein sequences, reflecting the lack of genetic information on fungi generally and more specifically on mycoparasites. It is therefore likely that some of the sequences represent newly discovered genes. Overall the number of novel sequences found here suggests that mycoparasitism in *S. elegans* is likely to involve many more genes than so far described in this study.

Among the 261 cDNA sequences, 120 unique ESTs were identified of which 95 appeared only once and 25 were represented by multiple sequences at frequencies ranging from 2 to 47. Nearly 46.1% of the sequences were related to pathogenesis including those related to toxin synthesis, pathogenic processes, stress response, multidrug resistance, and apoptosis. Consistent with recent genomic approach-based studies on *T. harzianum* (Liu and Yang, 2005) and *T. hamatum* (Carpenter et al., 2005), we identified several sequences (13%) corresponding to homologues of proteins required for synthesis and regulation of mycotoxins such as aflatoxins and trichothecenes in several fungi. Clone 2557, a homologue of the cytochrome P450-type monooxygenase of *Asperigillus fumigatus* and of the trichothecene C-15 of *Fusarium sporotrichioides*, could be involved in trichothecene biosynthesis, a mycotoxin produced by *S. chartarum* (Brasel et al., 2005), *T. brevicompactum* (Nielsen et al., 2005) *Fusarium* spp. (Brown et al., 2004), and *A. flavus* (Bhatnagar et al., 2003). Cytochrome P450-type monooxygenase together with the MFS (major facilitator superfamily) hexose transporter (EST 748), the transcription factor $Zn(II)_2Cys_6$ (EST 4522), and the O-methyltransferase B (EST 1729) are known to be involved in aflatoxin biosynthesis pathway in *A. parasiticus* and *A. flavus* (Bhatnagar et al., 2003; Ehrlich et al., 1999; Flaherty and Payne, 1997). In addition, domain searches showed that acetylcholinesterase (ESTs 3340 and 6358) are a part of the α/β

hydrolase superfamily associated with antifungal properties from *Aspergillus niger* (Bourne et al., 2004). Taken together, our results suggest that genes encoding proteins related to mycotoxin production could be involved in the mycoparasitic attack of *R. solani*.

Several upregulated genes representing 35.9% were predicted to encode a variety of functions involved in pathogenic processes, including those that make up components of the cellular machinery and those required for pigment synthesis and melanization processes. These include two genes homologous to short-chain dehydrogenase/reductases from *Cryphonectria parasitica* that belongs to oxydoreductases. This family of proteins is associated with different pathogenic processes in fungi (Fornarotto et al., 2006; Oppermann et al., 2003). The Ctr copper transporters (EST 2089) are essential for pigment and melanin synthesis in fungi (Petris, 2004). Genes encoding ferric-chelate reductases (ESTs 2791, 5053 and 1042) are known to mediate iron uptake and acquisition during pathogen growth by limiting this ion to their hosts (Zarnowski and Woods, 2005). This enzyme could be involved in providing nutrition for *S. elegans* from host cell components. Homologous to ankyrin repeat protein of *Aspergillus fumigatus*, EST 1867 is associated with the F-box motif that is required as a pathogenicity factor of *Fusarium oxysprum* on tomato (Duyvesteijn et al., 2005). It could be that EST 1867 is required as a pathogenicity factor during mycoparsitism, but its involvement requires further examination.

EST 4645 was identified based on its homology with *Neurospora crassa*. Calmodulin. Calmodulin is postulated to be involved in pathogenic processes (Kraus and Heitman, 2003), including the development of appressorium and stress responses in the fungal pathogens, *Magnoprothe grisea* and *Colletotrichum trifolii* (Liu and Kolattukudy, 1999; Warwar and Dickman, 1996; Warwar et al., 2000), and in the biosynthesis of aflatoxin in *Aspergillus* spp. (Juvvadi and Chivukula, 2006). The mycoparasitic interaction between *S. elegans* and *R. solani* involves morphogenetic processes that result in the formation of specific structures including hyphal coil, and appressoria or penetration pegs (Benyagoub et al.,

1994). Thus, it is not surprising to have the calmodulin gene preferentially expressed during an attack on *R. solani.*

Nearly 8% of the genes were predicted to encode membrane proteins involved in intracellular transport processes of eukaryotic cells (Baker et al., 2001; Calero et al., 2001). These include EST 2065 homologous to Yop-1 protein in *N. Crassa* and EST 5977 homologous to Mog1p in *Candida albicans.* Overexpression of *Yop1p* in *Saccharomyces cerevisiae* negatively regulates cell growth resulting in accumulation of internal cell membranes and a block in membrane traffic (Calero et al., 2001). During very early stages of *S. elegans* with an actively growing *R. solani* culture, conidial germination is substantially delayed by 30 h compared to that occurring in the absence of a host (Morissette et al., 2006). This delay could be due to the presence of inhibitory molecules released by *R. solani* shortly after exposure to *S. elegans.* In this study, we speculate that the up-regulation of *Yop-1* in the tester cDNA populations could be an indicator of cell growth regulation in *S. elegans* prior to its encounter and invasion of *R. solani.*

As a component of the genetic machinery that regulates the synthesis of ribosomal constituents, the increase in expression of genes encoding ribosomal proteins during mycoparasitism may be due to an increase in the synthesis of mycoparasitism-related proteins. ESTs 6535 and 2599 show similarity to fungal 60S L10 and L12 ribosomal proteins, respectively. The regulation of these genes during mycoparasitism parallels that of the *T. hamatum* gene encoding the 60s L36 cytoplasmic ribosomal protein which is up-regulated during confrontation with *Botrytis cinerea* (Fekete et al., 2001). In addition, many ESTs homologous to fungal mitochondrial rRNAs were preferentially expressed, and the majority of these genes presented a signal peptide or a tonB dependent receptor signature suggesting that the corresponding proteins are excreted or anchored in membranes. There is convincing evidence indicating that rRNAs have extraribosomal functions (Shi et al., 2004; Wool, 1996). Gene expression in prostate-cancer cell lines using SSH showed that several preferentially up-regulated ESTs belonged to rRNA, suggesting that these proteins play a role in

pathogenicity (Vaarala et al., 1998). Whether the rRNAs in our study play a similar role during mycoparasitism needs to be investigated.

Interestingly, none of the sequenced genes matched those encoding cell wall-degrading enzymes (CWDEs). This finding greatly differs to what had been reported on EST and proteomic analyses of *Trichoderma* species (Grinyer et al., 2005; Liu and Yang, 2005). Although not expected, the lack of genes encoding CWDEs could be attributed to the fact that our genomic-based approach (SSH and differential screening) was explored when *S. elegans* was grown in the presence of a live host at different time periods and not when it was grown in the absence of its host (Liu and Yang, 2005) or on a carbon source (Grinyer et al., 2005). Our approach led to the enrichment and discovery of genes that are not related to cell wall lytic processes, and established that active mycoparasitism stimulated the expression of genes of various functions.

In this study, the majority (77.5%) of the characterized genes are expressed in the mycoparasite, while 12.5% of the remaining genes belong to *R. solani.* Two redundant ESTs (5146 and 3391) notably matched pyridoxal reductase ARK8 from *Aspergillus fumigatus* that is involved in the production of vitamin B6, an essential nutrient for the growth of several fungi (Morita et al., 2004). The overexpression of AKR8 could be a response of *R. solani* in order to resist the attack by *S. elegans.* The genes that were identified to be involved in the respiration chain were all expressed by *R. solani.* The 40S ribosomal protein S9 (EST 4552) is a mitochondrial protein that interferes with mitochondrial function when it is over expressed (Wiltshire et al., 1999). This could be the result of the activity of the mycoparasite.

Similar to *ubiquitin 1* in *Gibberella pullicaris*, this gene (EST 2380) was overexpressed in both organisms. This is not surprising since ubiquitins are known to play a role in both cellular stress response and protein degradation in eukaryotes including fungi (Welchman et al., 2005). The presence of ubiquitin in the host could be related to stress in response to attack by *S. elegans*, while ubiquitin in *S. elegans* may play a role in protein degradation during its growth inside the host.

In conclusion, this study allowed the isolation of several genes that are overexpressed during *in vivo* mycoparasitic interaction. These genes present a broad range of functions reflecting the complexity of the genetic regulation during this process. With more expression and genetic experiments, these genes provide new insights into the current understanding of the mycoparasitic process. For the first time, defense-related genes from the host *R. solani* were isolated. Moreover, several novel genes from the mycoparasite were isolated. Three unidentified genes showed an important up-regulation of their expression during interaction of the two fungi, suggesting genes other than those encoding CWDEs could be a target to improve mycoparasitic activity and plant resistance. Finally, the results suggest that *S. elegans* produces mycotoxins during mycoparasitism, which is new for this species.

5.6. ACKNOWLEDGMENTS

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CONNECTING STATEMENT BETWEEN CHAPTERS 5 AND 6

Several genes that were upregualted during the mycoparasitism of *Rhizoctonia solani* by *Stachybotrys elegans* were isolated and partially characterized in Chapter 5. The current chapter describes the expression pattern over time of eight newly characterized genes and the endochitinase-encoding gene *sechi44*, all expressed by *S. elegans*, using QRT-PCR. The expression analysis of these genes was conducted when the mycoparasite was in confrontation with the host's (*R. solani*) and non-host's (*Sclerotinia sclerotiorum*) hyphae and sclerotia. In the conidia-hyphae interaction experiment, the conidia of *S. elegans* were sprayed on the growing hyphae of the host and non-host, and RNA was extracted at different time points. In the conidia-sclerotia interaction experiment, dead and live sclerotia of the host and non-host were imbibed with conidia suspension and RNA was extracted at different time points. The temporal and differential relative gene expression was calculated using Pfaffl method (Pfaffl, 2001) based on three biological replicates and two technical replicates and efficiencies were calculated using Liu and Saint methods (Liu and Saint, 2002). The statistical analyses were conducted using two-way ANOVA. The comparison of expression on host and non-host allowed us to evaluate the importance of selected target genes in mycoparasitism.

The results of this section will be submitted for publication in Phytopathology. I designed all the experimental set-up except the conidia-sclerotia interaction, conducted all the experiments, and wrote the manuscript. The contributions of the co-authors were as follows: Professor S. Jabaji-Hare provided supervision, and funding throughout this study. She also made suggestions and corrected the manuscript. Monique Arts, M.Sc. candidate in Dr. S. Jabaji-Hare's laboratory, designed the experimental set-up of the conidia-sclerotia interaction, treated hyphae and sclerotia, extracted RNA and retro-transcribed RNA into cDNA, and quantified the expression of the calmodulin and *sechi44* genes. Professor P. Séguin helped with the choice of statistical methods, analysis of data, and revised the final version of the manuscript.

CHAPTER 6

Expression analysis of novel mycoparasitism-related genes.

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6.1. ABSTRACT

Transcript patterns elicited in response to hosts can reveal how a mycoparasite recognize suitable hosts and the mechanisms involved in pathogenicity. Temporal and differential gene expression of nine mycoparasitism-related genes was monitored during the interactions between *Stachybotrys elegans* and hyphae and sclerotia of the host *Rhizoctonia solani* and the non-host *Sclerotinia sclerotiorum*. Using real-time reverse transcription-polymerase chain reaction, comparative analyses demonstrated that hyphal and sclerotial forms triggered different expression pattern and the presence of host and non-host caused different gene regulation. Some genes such as *seglu*, *selec*, and *se151* were completely inhibited by the presence of non-host hyphae suggesting that these genes play an important role during mycoparasitism. Also, the absence of these corresponding transcripts suggests that the non-host produces transcription inhibitors. As expected, gene expression of *cytochrome P450* was highly up-regulated early after germination of *S. elegans* conidia. This is in agreement with our finding on EST data mining study, in which a role in toxin production was assigned to *cytochrome P450* (Morissette et al., personal communication). In this study, only the expression of some genes such as, *acetylcholinesterase*, *calmodulin*, and *sechi44* were influenced by the structural anatomy of the sclerotia suggesting that a component of the sclerotia stimulates the expression of these genes.

KEYWORDS: mycoparasitism-related genes, QRT-PCR, gene expression, mycoparasite

6.2. INTRODUCTION

Stachybotrys elegans is a filamentous fungus commonly found in soil that displays biocontrol capabilities against *Rhizoctonia solani* AG-3 (Benyagoub et al., 1994), a known fungal pathogen of potato. The mycoparasitic properties of *S. elegans* enable it to colonize its host by accomplishing several successive steps: recognition and production of fimbrial extracellular matrix that surrounds the host cell (Benyagoub et al., 1994; Benyagoub et al., 1996), coiling of the hyphae, and the formation of appressoria that aid in penetrating the host cell wall followed by complete intracellular colonization leading to destruction of hyphae and sclerotia of *R. solani* (Benyagoub et al., 1994). This process is accompanied by the secretion of cell wall-degrading enzymes (CWDEs) including chitinases (Morissette et al., 2003; Taylor et al., 2002; Tweddell et al., 1995), glucanases (Archambault et al., 1998b), and cellulases (Tweddell et al., 1995), which are understood to directly attack the cell of *R. solani*, causing cell wall lysis and death (Benyagoub et al., 1994). An endochitinase gene, *sechi44*, was previously cloned and characterized from *S. elegans* (Morissette et al., 2003). Evidence for its participation in the mycoparasitic process of *R. solani* hyphae and in the growth of *S. elegans*, its stimulation by purified host cell wall fragments, and regulation by nitrogen and carbon availability was recently provided (Morissette et al., 2006).

Most research on mycoparasitism has focused on the model biocontrol strains of *Trichoderma* species and much understanding on the role of selected CWDEs enzymes and some extracellular proteases and their encoding genes has been gained on these strains (Delgado-Jarana et al., 2002; Grinyer et al., 2005; Pozo et al., 2004; Suarez et al., 2005). Recent analysis of gene expression in mycelium of *T. harzianum* using an expressed sequence tag (EST) approach (Liu and Yang, 2005) identified several clones. However, these clones did not reflect the genetic regulation of the mycoparasitic interaction with a living host and its defense reaction, but reflected gene regulation in the presence of a cell wall preparation. Despite these advances, we still have not observed the total diversity of other enzymes and their encoding genes from other mycoparasites, nor do we

have comprehensive studies on the genetic regulation of these proteins during mycoparasitism.

By understanding the basic mechanisms of action and regulation of genes involved in mycoparasitism of *R. solani* by *S. elegans*, the development of approaches for detecting and reducing the impact of *R. solani* or increasing the biocontrol activity of *S. elegans* may be achieved. This led us to initiate a large-scale EST study and we characterized 261 ESTs of *S. elegans* and *R. solani* that were preferentially upregulated during mycoparasitism. Based on our findings, the majority of these genes were not identified nor reported previously to be involved in mycoparasitism, thus making them potential targets for gene regulation studies during this process. Among those expressed by *S. elegans*, three uncharacterized ESTs identified as having multiple functions (DW520881 and DW520691) or no match (DW520687) and five ESTs encoding the following: *acetylcholinesterase*, recently associated with antifungal properties in *Aspergillus niger* (Bourne et al., 2004), *ankyrin repeat protein*, the most common protein-protein interaction motif in nature (Mosavi et al., 2002), associated with another motif, the F-box protein, which is required for the pathogenicity of *Fusarim oxysporum* on tomato (Duyvesteijn et al., 2005), *calmodulin* whose role besides signalling, has been recently implicated in the control of virulence and the development of appressoria in *Colletotrichum trifolii* and *Magnaporthe grisea*, two important plant pathogens (Kraus and Heitman, 2003; Liu and Kolattukudy, 1999; Warwar and Dickman, 1996; Warwar et al., 2000), *cytochrome P450* whose role is related to the biosynthesis of the mycotoxin trichothecene (Alexander et al., 1998; van den Brink et al., 1998), and *mog1* a nuclear protein that interacts with *Ran*, and confers directionality to nuclear import and export pathways (Baker et al., 2001). The overexpression of the above genes in response to parasitizing *R. solani* hyphae was confirmed in RNA samples pooled from 8 time points (D. Morissette and S. H. Jabaji-Hare, unpublished observations).

No comprehensive description of the temporal transcription of mycoparasitism-related genes is available when a mycoparasite colonizes hyphae and sclerotia of different sclerotial forming fungi. We selected the structurally-

defined tuberoid sclerotia of *Sclerotinia sclerotiorum* (Willetts and Bullock, 1992) because of the differences in the anatomy and size of the sclerotia compared to those of *R. solani*, which consist of a loose network of hyphae and the absence of a rind. Based on our EST analysis and the involvement of the identified genes in pathogenic processes and toxin metabolism in other fungi, the current study was conducted to investigate whether the expression pattern over time of these novel mycoparasitism-related genes are altered when *S. elegans* interacts with hyphae and sclerotia of its host, *R. solani*, and a non-host, *Sc. sclerotiorum,* and whether the differences in the structure or the chemical composition of sclerotia might affect the transcription of the genes. To do so, we monitored the temporal gene expression of these genes using quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR).

6.3. MATERIALS AND METHODS

6.3.1. Fungal strains and growth conditions.

S. elegans (ATCC 188825) and *R. solani* AG-3 (ATCC 10183) starter cultures were grown from precolonized oat kernels on 1% potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) and incubated at 24°C for 7 and 5 days, respectively. *Sc. sclerotiorum* (isolate SS5b, isolated from lettuce and supplied by A. Watson, McGill University, Qc) starter cultures were grown from sclerotia on 1% PDA for 5 days at 24°C.

6.3.2. Preparation of *S. elegans* conidia and host and non-host sclerotia.

In order to induce conidiation of *S. elegans*, starter cultures were homogenized in a blender (Model 31BL92, Warning) for 4x2 s pulses with sterile double-distilled water. One ml of the homogenate was spread on a permeable cellophane membrane (500 PUT; UCB, North Augusta, USA) that was placed on 1% PDA culture plates, and incubated at 24°C. After 7 days, mycelia were harvested by carefully scraping the surface of the membrane, and gently shaken in sterile water to dislodge the conidia. The mycelia were removed from the suspension by filtering through a sterile nylon cloth. The filtrate was centrifuged

at 3000 rpm for 10 min and the conidial pellet was then resuspended in sterile water and kept on ice until further use. Depending on the type of experiment, the concentration was adjusted to 10^6 conidia/ml or 10^7 conidia/ml using hematocytometer (s/p ULTRAPLANE Spot lite counting chamber; Improved Neubauer 1/400 sq. mm, 1/10 mm deep). Conidia were applied to the respective treatments immediately after the concentration was adjusted.

To harvest sclerotia of host and non-host fungi, agar plugs (6 mm) from 5-day old *R. solani* and *Sc. sclerotiorum* starter cultures were placed on cellophane-covered PDA. Cultures were incubated at 24°C for 25 days, allowing sufficient development of sclerotia. Uniform sized sclerotia (5 mm in size) of host and non-host were selected and air-dried. To inactivate the sclerotia for the "dead" treatments, half of both types of sclerotia were autoclaved for 20 min at 121°C.

6.3.3. Interaction of *S. elegans* with mycelia of host and non-host.

In order to evaluate if changes in target gene expression are attributable to the mycoparasitic activity (i.e. interaction of the mycoparasite with its host) or due to the simple presence of another fungus (even a non-host), expression of *S. elegans*' genes in the presence of its host (*R. solani*) or a non-host (*Sc. sclerotiorum)* was monitored at different time points during dual culture interactions. Dual culture assays were carried out in the absence of light on 100x15 mm culture Petri plates containing minimal synthetic medium (MSM; Tweddell et al., 1995) supplemented with 1% Gellan Gum (Kelco, San Diego, USA) and covered with a permeable cellophane membrane (Morissette et al., 2003). The experimental set-up consisted of agar plugs (6 mm) from the respective fungi placed on the surface of the cellophane-covered solid MSM plates. The plates were incubated at 24°C, allowing *Sc. sclerotiorum* and *R. solani* cultures to grow for three and four days, respectively. The cultures were then sprayed with 100 µl of the 10^6 conidia/ml suspension, using a Badger 350 Air-Brush and MC-80 Mini Air Compressor at 1 kg/cm^2, and incubated at 24°C. The control treatment consisted of spraying 100 µl of conidia of *S. elegans* on cellophane covered MSM plates. Total mycelia from each plate were harvested

after 48, 66, 72, 84, 96, and 108 h of treatment. This set-up allowed the conidia of the mycoparasite to sporulate and grow in contact with the hyphae of the host or non-host, and eventually interact with each other. The use of the membrane facilitated the removal of the fungi from the plate for subsequent RNA analysis. There were three replicates for each treatment and harvesting time. Mycelia were completely harvested from each plate at the designated times, flash frozen in liquid nitrogen, and stored at -80°C.

6.3.4. Interaction of *S. elegans* with host and non-host sclerotia.

To investigate if the expression of *S. elegans* target genes is altered in the presence of different types of live and dead sclerotia, gene expression was monitored at different time points after inoculation of host and non-host sclerotia with *S. elegans* conidia. Each treatment consisted of 5 sclerotia from the respective fungus placed on a cellophane membrane on solid MSM in a 100x15 mm Petri plate. Each sclerotium was inoculated with 30 µl of the 10^7 conidia/ml suspension, for a total of 30 000 conidia per sclerotium or 150 000 conidia per plate. All plates were incubated at 24°C and sclerotia were collected after 3, 5, 7, and 12 days, immediately immersed in liquid nitrogen, and stored at -80°C. There were three replicates for each treatment and harvesting time.

6.3.5. RNA extraction and retrotranscription (RT).

All fungal samples were ground into powder using a mortar and pestle. Total RNA was extracted from 100 mg of powdered tissue using RNeasy Plant Mini Kit (QIAGEN Inc., Mississauga, ON) following manufacturer's recommendations. RNA integrity and concentration were assessed by spectrophotometry using a Nanodrop ND1000 (NanoDrop, Wilmington, DE), and by 1.2% formaldehyde-agarose gel electrophoresis. A total of 500 ng of RNA was reverse-transcribed using Quantitech Reverse Transcription Kit (QIAGEN) following the manufacturer's recommendations, and DNA present in the samples was destroyed using the DNase Wipeout Buffer included in the kit. cDNA integrity was verified by conventional RT-PCR using the universal primers ITS1F

and ITS4 (Gardes and Bruns, 1993; White et al., 1990; Table 6.1), followed by electrophoresis and visualization on a 1% agarose gel. The transcribed cDNA was diluted 1/20 in sterile water to reduce effects of interference by RT reaction components in downstream PCR applications.

6.3.6. Primer design.

Primer pair sets for eight target genes and one housekeeping gene (HKG) were designed with the software Primer 3 (Rozen and Skaletsky, 2000) and submitted to Nucleotide BLAST at NCBI to confirm specificity (Table 6.1), and were custom synthesized by AlphaDNA (Montréal, QC). Except for *sechi44* and the HKGs, all primer sets were designed based on ESTs previously identified from a subtractive library constructed from *S. elegans-R. solani* interaction (Genbank accession numbers : DW520693, DW520683, DW520690, DW520689, DW520686, AF516397, DW520881, DW520691, DW520687). The primers sec1R and sec1F were designed by Morissette et al. (2006) in order to amplify a segment of the endochitinase-encoding gene *sechi44* from *S. elegans*. The HKGs encoding histone-3 and histone-4 were amplified with primer pairs H3-1a and H3-1b and H4-1a and H4-1b (Glass and Donaldson, 1995), respectively from *S. elegans* only. Primers of another potential *S. elegans* HKG, elongation factor 1 (*EF1*), was also designed. However, histone-4 and EF1 primer pairs were unsuitable to be used in QRT-PCR reactions since they amplified gene products in *Sc. sclerotorium*.

All primers were tested on *S. elegans*, *R. solani*, and *Sc. sclerotiorum* genomic DNA and cDNA in (RT)-PCR assays, followed by gel electrophoresis to confirm the fungal origin of the amplified products. Melting point analyses were systematically performed at the end of the QRT-PCR to confirm the amplification of a unique product for each of the target genes and the HKGs.

6.3.7. Rapid amplification of cDNA ends (RACE-PCR) of *seglu*, *selec*, and *se151* genes.

In order to identify the uncharacterized genes *seglu*, *selec*, and *se151* (GenBank Accession numbers: DQ369849, DQ369850, DQ872900; Appendix I), RACE-PCR was conducted using GeneRacer™ kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. One microgram of total RNA previously extracted from the dual culture of *S. elegans* and *R. solani* was used (Morissette et al., 2006). The sequencing was performed at the Genome Quebec Innovation Center (McGill University, Montreal, Qc, Canada) using the M13 universal primers. Sequence analysis was carried on with Chromas 2.3 (http://www.technelysium.com.au/chromas.html), DNAman (version 4.0), VecScreen (NCBI), and translate (ExPASy). Sequences were then analyzed for conserved domains using standard blast (n and x) analysis Altshul 1997 on all sequences registered in NCBI database, InterProScan (http://www.ebi.ac.uk/InterProScan/), and SMART (smart.embl-heldelberg.de; Schultz et al., 1998; Letunic et al., 2006).

6.3.8. Expression analysis by QRT-PCR.

QRT-PCR was performed for each of the target genes and for the HKG *histone-3* using Mx3000 (Stratagene, Cedar Creek, USA) and SYBR Green master mix (Stratagene) following the manufacturer's recommendations. Amplification was performed in a 24-µl reaction mixture containing the 125 nmol of each primer (250 nmol for selec, 375 nmol for se151), 1x SYBR Green master mix, 30 nmol of reference dye ROX, and 2 µl of cDNA template ($1/20^{th}$ dilution of total cDNA). Thermocycling was performed at an initial denaturation temperature of 95°C for 10 min (hot start), followed by different conditions according to the amplified gene: 50 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 11 s, with fluorescence reading was done at 76°C for 11 s for *acetylcholinesterase*; 50 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 11 s, with fluorescence reading at 72°C for 11 s for *ankyrin repeat protein*; 40 cycles of 95°C for 30 s, 63°C for 45 s, and 72°C for 11 s, and fluorescence reading at 72°C

at the end of the elongation cycle for *calmodulin*; 50 cycles of 95°C for 30 s, 66°C for 45 s, and 72°C for 11 s, for *cytochrome P450*, *mog1*, and *selec*, and with fluorescence reading at 80°C for 11 s for *cytochrome P450*, 81°C for 11 s for *mog1*, and at 72°C at the end of the elongation cycle for *selec*; 45 cycles of 95°C for 30 s, 66°C for 1 min, and 72°C for 25 s, and the reading of fluorescence was done at 77°C for 11 s for *sechi44*; 45 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 11 s, and fluorescence reading at 72°C at the end of the elongation cycle for *seglu*; 45 cycles of 95°C for 30 s, 69°C for 45 s, and 72°C for 11 s, and the reading of fluorescence was done at 72°C at the end of the elongation cycle for *se151*; 50 cycles of 95°C for 30 s, 67°C for 45 s, and 72°C for 30 s, and the reading of fluorescence was done at 72°C at the end of the elongation cycle for *histone-3*. Finally, a melting curve was generated (55-95°C with a heating rate of 0.1°C/s). Each run included a negative control was repeated twice on two different days. Data were presented as averages of the two technical replicates.

In gene expression studies, the terminology used to describe transcript level alteration is sometimes confusing. Terms such as gene induction and stimulation or inhibition and repression are respectively used interchangeably. In this study, we followed the pattern of expression over time and among treatments, and therefore we refer to the term induction when a gene is upregulated and repression when gene expression is down regulated.

6.3.9. Data quantification.

Quantification was based on a generated amplicon using the different gene-specific primer pairs. Data generated by QRT-PCR were estimated using Stratagene analysis software. QRT-PCR reaction for each sample was repeated twice (technical replicates). QRT-PCR data were calculated as a normalized relative expression of gene using the equation [6.1] developed by Pfaffl (2001), based on crossing point (CP) and efficiency obtained for the HKG *histone-3* and the different target gene amplifications.

$$(E_{ref})^{CP}/(E_{target})^{CP} \qquad [\,6.1\,]$$

E is the PCR efficiency calculated with the equation [6.2], E_{ref} is the efficiency of the reference gene (HKG, histone-3), and E_{target}, the efficiency of the target gene. CP is the crossing point taken at an arbitrary threshold of 0.02.

$$E = (R_{n,B} / R_{n,A})^{[1/CP_B - CP_A]} \qquad [6.2]$$

Equation [6.2] shows a mathematical model for the calculation of the efficiency of each amplification curve in which $R_{n,A}$ and $R_{n,B}$ are reporter fluorescence at cycle n at arbitrary thresholds A and B, CP_A and CP_B are the crossing points at these arbitrary thresholds (Pfaffl personal communication; http://www.gene-quantification.info/).

The relative expression of each gene was tested for significant difference between harvesting time and treatments by two-way analysis of variance (ANOVA) using the software SAS (SAS institute inc., Cary, NC, USA, version 8.2). Comparisons between means at each time point were made using least significant differences (LSD) at $P < 0.05$.

6.4. RESULTS

6.4.1. Characterization of the genes *seglu*, *selec*, and *se151*.

The full length of *seglu* cDNA is 1389 bp, and the deduced SEGLU protein sequence shows an open reading frame (ORF) of 280 amino acids (aa) with a signal peptide spanning from residue 1 to 19. SEGLU has a predicted molecular weight (MW) of 31943.41 Da and an isoelectric point (pI) of 5.89. Blastn showed several homologies with distinct sequences only for the 40 first nucleotides. Blastx showed homology with hypothetical proteins from *Mycobacterium avium* (e^{-5}) and *Gibberella zeae* (e^{-4}). The full length of *selec* cDNA is 524 bp and is encoded in the mitochondrial DNA. The deduced protein SELEC showed an ORF of 115 aa with a signal peptide and two transmembrane regions. SELEC has a predicted MW of 13243.44 Da and pI of 9.54. Blastn showed several homologies with distinct sequences only for the first 36 nucleotides. Only the 3'RACE-PCR has been successfully sequenced for *se151*.

The length of the partial cDNA of *se151* was 838 bp. Blastn and Blastx did not reveal any significant homology. Even if the 5'RACE-PCR was not successful, it is likely that the entire ORF is present in the partial cDNA sequence. Thus, the SE151 deduced peptide sequence is 218 aa length, with a predicted MW of 24648.18 Da and an pI of 8.49. None of the three genes are associated with known domains.

6.4.2. Gene expression during hyphal interaction with a host and a non-host.

The temporal expression of all genes varied significantly ($P < 0.05$) when *S. elegans* conidia were grown with or without a host or non-host (Table 6.2). Generally, the expression of the majority of target genes was higher in the presence of the host rather than non-host. Most notable was the gradual increase in *cytochrome P450* expression at 66 h and onwards reaching 17.5-fold at 108 h (Table 6.2). Compared to the control, there were significant changes in the expression of some genes as early as 48 h of interaction. An induction of *cytochrome P450 (*33.0 fold) and *mog1* (3.0 fold), and a repression of *ankyrin repeat protein* and *seglu* was observed (Table 6.2). At 108 h, generally there was no change in gene expression except for a 6.0-fold induction for *cytochrome P450* and a 3.5-fold repression of *mog1* (Table 6.2). Interestingly, in the presence of the host, the transcription of *selec* was significantly repressed across all time points, with the greatest decrease observed at 48 h (340 fold), diminishing to 25 fold at 84 h (Table 6.2). No significant change in *calmodulin* and *se151* expression was observed over the duration of the experiment (Table 6.2), compared to that observed in *S. elegans* alone.

In the presence of the non-host, the expression of three genes was repressed at all time points (*seglu*, *selec*, and *se151*) or at specific time points (*acetylcholinesterase, ankyrin repeat protein*, and *cytochrome P450*). However, in the case of *mog1* and *calmodulin*, the non-host substantially induced their expression at 72 h by 6.0 fold and at 108 h by 8.0 fold, respectively, compared to when the mycoparasite was grown alone or in the presence of its host (Table 6.2).

6.4.3. Gene expression during interaction with sclerotia of a host and a non-host.

Irrespective of the treatment, the expression of all genes was affected over time ($P< 0.05$) (Figs 6.2-6.5). In live host sclerotia, the expression of all genes was altered over time except for *ankyrin repeat protein* and *se151* (Fig. 6.2). The most striking increase was observed for *calmodulin* between 3 and 7 days (6.6 fold) and *sechi44* between 3 and 12 days (430.8 fold) (Figs 6.2C, F), while a gradual decrease of expression was observed for *mog1* and *seglu* between 3 and 12 days (2.7 fold and 3.1 fold), and *selec* between 3 and 7 days (5.4 fold; Figs 6.2E, G, and H). In contrast, with live non-host sclerotia, expression of all genes was not altered over time except for *acetylcholinesterase*, *ankyrin repeat protein*, and *se151* (Fig. 6.2A, B, I).

6.4.3.1. Live host sclerotia vs. live non-host sclerotia.

Infection of live host compared to live non-host sclerotia highly induced ($P < 0.05$) the expression of the target genes at various time points (Fig. 6.2), with the highest fold induction in transcription observed at 7 days for *cytochrome P450* (71.2 fold) and *calmodulin* (6.2 fold; Figs. 6.2D and C), at 3 days for *mog1* (4.1 fold), *seglu* (29.6 fold), and *selec* (9.7 fold; Figs. 6.2E, G and H), and at 12 days for *sechi44* transcription (7.6 fold; Fig. 6.2F). On the other hand, interaction with the non-host induced the expression of some genes; *sechi44* (144.7 fold) as early as 3 days of infection, *ankyrin repeat protein* at 5 days (4.8 fold), and *se151* (2.5 fold) at 12 days (Figs 6.2B, F, I).

6.4.3.2. Dead sclerotia vs. live sclerotia.

Generally, when expression was altered, live sclerotia triggered a higher expression of the target genes than dead sclerotia (Figs 6.3 and 6.4). Effectively, a higher induction of *seglu* (5.4 fold; Fig. 6.3G) and *selec* (2.0 fold; Fig. 6.3H) as early as 3 days of interaction, *cytochrome P450* at 5 and 7 days (3.3 and 42.7 fold respectively; Fig. 6.3D), and *calmodulin* (3.3 fold; Fig. 6.3C) and *sechi44* (3.5 fold; Fig. 6.3F), at 12 days was observed with live host as compared to dead host

sclerotia. In contrast, the expression of *acetylcholinesterase* (4.0 and 2.2 fold) and *calmodulin* (5.7 and 2.3 fold) was repressed after 3 and 5 days of interaction (Figs 6.3A and C), while the expression of *cytochrome P450* (Fig. 6.3D) was repressed at 3 days. The presence of live non-host sclerotia compared to dead non-host sclerotia induced the expression of *acetylcholinesterase*, *ankyrin repeat protein*, and *se151* (Fig. 6.4) at different time periods with the highest fold induction in transcription observed at 5 days for *ankyrin repeat protein* (5.7 fold; Fig. 6.4B). Expression of the remaining genes was not affected (data not shown).

6.4.3.3. Dead host sclerotia vs. dead non-host sclerotia.

The expression of *acetylcholinesterase*, *calmodulin*, and *sechi44* was induced at different time periods after the inoculation of dead host sclerotia compared to inoculated dead non-host sclerotia (Fig. 6.5). The type of sclerotia did not affect the expression of the other six remaining genes (data not shown).

Table 6.1. Primers designed and used in quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) for amplifying genes involved in the mycoparasitic process of *Stachybotrys elegans*.

Target	Acc. number	Primer	Sequence (5'- 3')	Amplicon size (bp)	Reference
Acetylcholinesterase	DW520693	Actyl-F	CTGCGGCCTTATTTAGAGACTG	112	This study
		Actyl-R	GACCAAGCATGTTCAGAAGTCC		
Ankyrin repeat protein	DW520683	Ank 1867-F	GACTCTATGGTGGTGGGTTAGA	113	This study
		Ank 1867-R	CTACCTCACTGGACTCATCGTT		
Calmodulin	DW520690	Calmod-F	CGGCAGAGATGAAACCGTTGTTGT	120	Morissette et al.,
		Calmod-R	TTGACTTCCCAGAGTTCCTGACCA		unpublished data
Cytochrome P450	DW520689	Cytc-F	AGATGCGAGTGGCGCAAGTTCTTT	137	Morissette et al.,
		Cytc-R	TTCGCAGCGACTCGAGAACCATTA		unpublished data
Mog1	DW520686	Mog-F	TCGATCAGGATGGTTTCACCAGCA	129	Morissette et al.,
		Mog-R	TGATATCGGTGCCAACCATGTCCT		unpublished data
sechi44	AF516397	Sec1-F	GACGCGGATATTGAGAAGCACTAC	128	Morissette et al.,
		Sec1- R	TGGACAGCATGATCTTGAGGTTT		unpublished data
seglu	DW520881	seglu-F	GAGAACAGCAGTCTTCATTTC	111	Morissette et al.,
		seglu-R	CTAAGTATAGCACCAGAGGCA		unpublished data
se151	DW520691	se151-F	AATACGCCATACAGCCGAGAGACA	110	Morissette et al.,
		se151-R	TCAAGACGCCCGATTGGTTCTGAT		unpublished data
selec	DW520687	selec-F	ACGCTGTCACGCATATACCAATCC	134	Morissette et al.,
		selec-R	TCTCGGGCCATCCTTGGAATACTT		unpublished data
Histone-3	DQ369854	H3-1a	ACTAAGCAGACCGCCCGCAGG	390	Glass and
		H3-1b	GCGGGCGAGCTGGATGTCCTT		Donaldson, 1995

Table 6.2 Gene relative expression of different genes during the interaction of *S. elegans* with host (*R. solani*) and non-host (*Sc. sclerotiorum*) hyphae at different time periods. The relative expressions were calculated from three replicates using the equation from Pfaffl (2001). Relative expressions for the same gene within the same row followed by a different lower case letter are significantly different at $P \leq 0.05$ according to LSD. Relative expressions for the same gene within the same column followed by a different capital letters are significantly different at $P \leq 0.05$ according to LSD. *Se* alone = *S. elegans* conidia sprayed on MSM, *Rs+Se* = *S. elegans* conidia sprayed on *R. solani* mycelia, *Scs+Se* = *S. elegans* conidia sprayed in *Sc. sclerotiorum* mycelia.

Gene	Time	Relative expression		
Acetylcholinesterase		*Se* alone	*Rs*+*Se*	*Scs*+*Se*
	48h	1.8 Bab	5.6 ABa	0.8 Bb
	66h	0.7 B	0.3 C	2.9 A
	72h	3.6 B	1.4 BC	2.3 A
	84h	2.8 Ba	5.7 Aa	0.0 Cb
	96h	9.7 Aab	5.1 ABb	0.2 Ac
	108h	1.8 B	3.1 ABC	0.9 A
Ankyrin repeat protein				
	48h	0.7 a	0.0 Bb	0.0 Bb
	66h	0.5 a	0.3 Aa	0.0 Bb
	72h	0.9 a	0.4 Aa	0.0 Bb
	84h	1.4	1.1 A	0.2 A
	96h	1.4 a	0.4 Aa	0.0 Bb
	108h	1.7 a	0.8 Aa	0.0 Bb
Calmodulin				
	48h	81.1	13.7 B	58.6 BCD
	66h	30.0	20.2 B	23.5 CD
	72h	58.8	57.2 B	26.5 D
	84h	147.9	196.9 A	165.7 B
	96h	76.6	73.4 AB	142.4 BC
	108h	131.0 b	65.0 Bb	1044.3 Ac
Cytochrome P450				
	48h	0.4 ABb	13.9 Ca	0.0 Bc
	66h	0.1 B	2.7 D	1.1 A
	72h	0.3 B	5.2 D	1.3 A
	84h	5.5 Ab	13.3 Ca	0.2 Ab
	96h	4.6 ABb	23.5 Ba	1.1 Ab
	108h	5.1 Ab	30.4 Aa	1.7 Ab

Gene	Time	Relative expression		
Mog		*Se* alone	*Rs*+*Se*	*Scs*+*Se*
	48h	13.4 b	41.1 Aa	5.9 Bb
	66h	10.5	6.0 B	3.9 Bb
	72h	15.1 b	11.2 Bb	70.8 Aa
	84h	23.9	9.9 B	12.3 Bb
	96h	12.6	8.6 B	1.8 Bb
	108h	28.2 ab	8.0 Bb	9.6 Bab
seglu				
	48h	0.3 a	0.0 b	0.0 b
	66h	0.2 a	3.0 a	0.0 b
	72h	1.5 a	0.6 a	0.0 b
	84h	1.6 a	0.1 a	0.0 b
	96h	0.9 a	0.8 a	0.0 b
	108h	1.2 a	0.9 a	0.0 b
selec				
	48h	37.4 Ba	0.1 Cb	0.0 c
	66h	16.6 Ca	3.4 Bb	0.0 c
	72h	18.4 Ca	1.0 Bb	0.0 c
	84h	63.4 Aa	2.6 Bb	0.0 c
	96h	19.8 Ca	5.0 Bb	0.0 c
	108h	26.5 BCa	20.0 Aa	0.0 b
se151				
	48h	33.3 a	85.6 a	0.0 b
	66h	10.1 a	4.0 a	0.0 b
	72h	24.3 a	15.4 a	0.0 b
	84h	42.7 a	24.6 a	0.0 b
	96h	13.0 a	6.1 a	0.0 b
	108h	12.3 a	16.7 a	0.0 b

Figure 6.1. Gene relative expression of different genes during the interaction of *S. elegans* with live host (*R. solani*) and non-host (Sc. *sclerotiorum*) sclerotia at different time periods. A, *acetylcholinesterase*; B, *ankyrin repeat protein*; C, *calmodulin*; D, *cytochrome P450*; E, *mog1*; F, *sechi44*; G, *seglu*; H, *selec*; I, *se151*. The relative expressions were calculated from three replicates using the equation from Pfaffl (2001). Capital and lower case letters on the curves represent significance $P \leq 0.05$, according to LSD, across time and between treatments, respectively. ●, host sclerotia inoculated with *S. elegans* conidia; ▽, non-host sclerotia inoculated with *S. elegans* conidia.

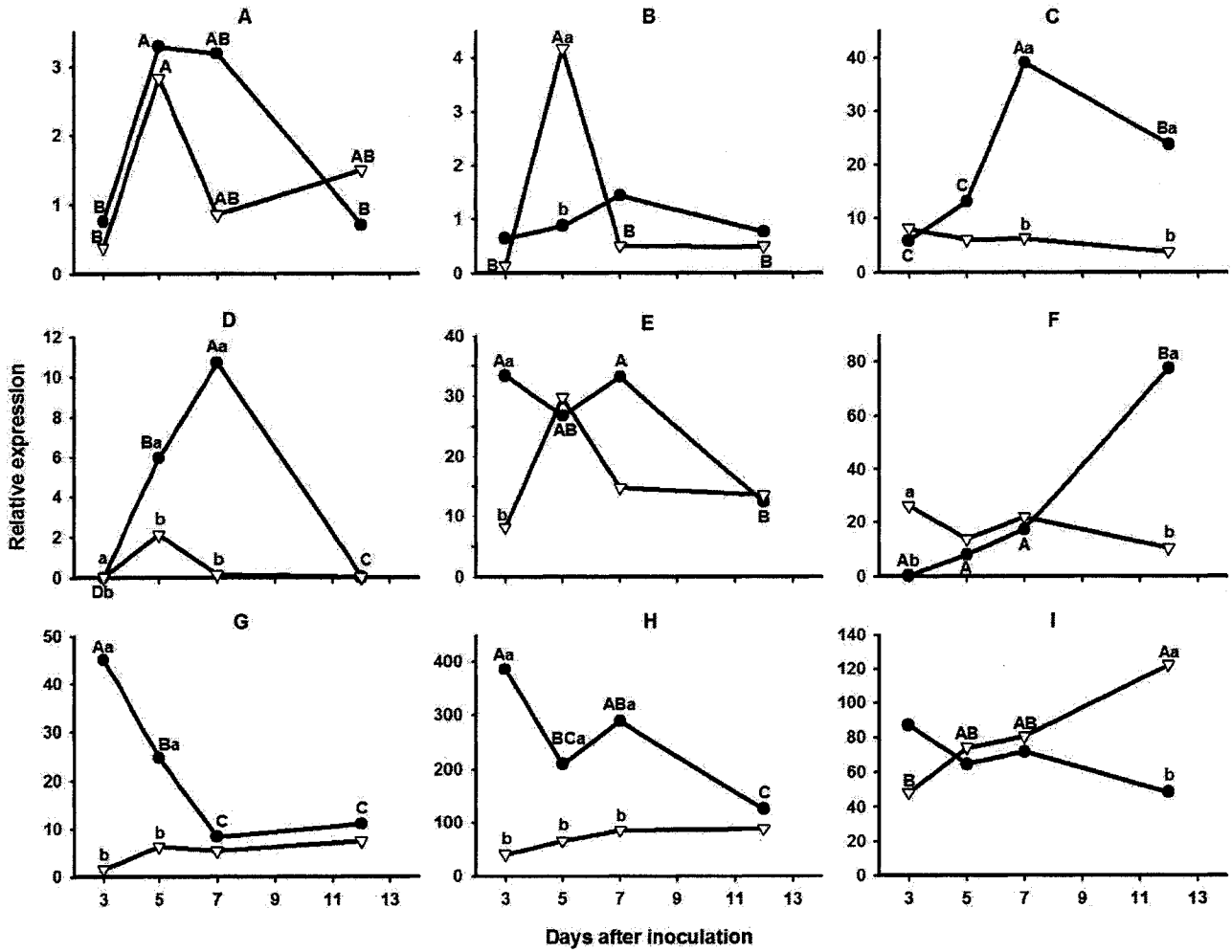
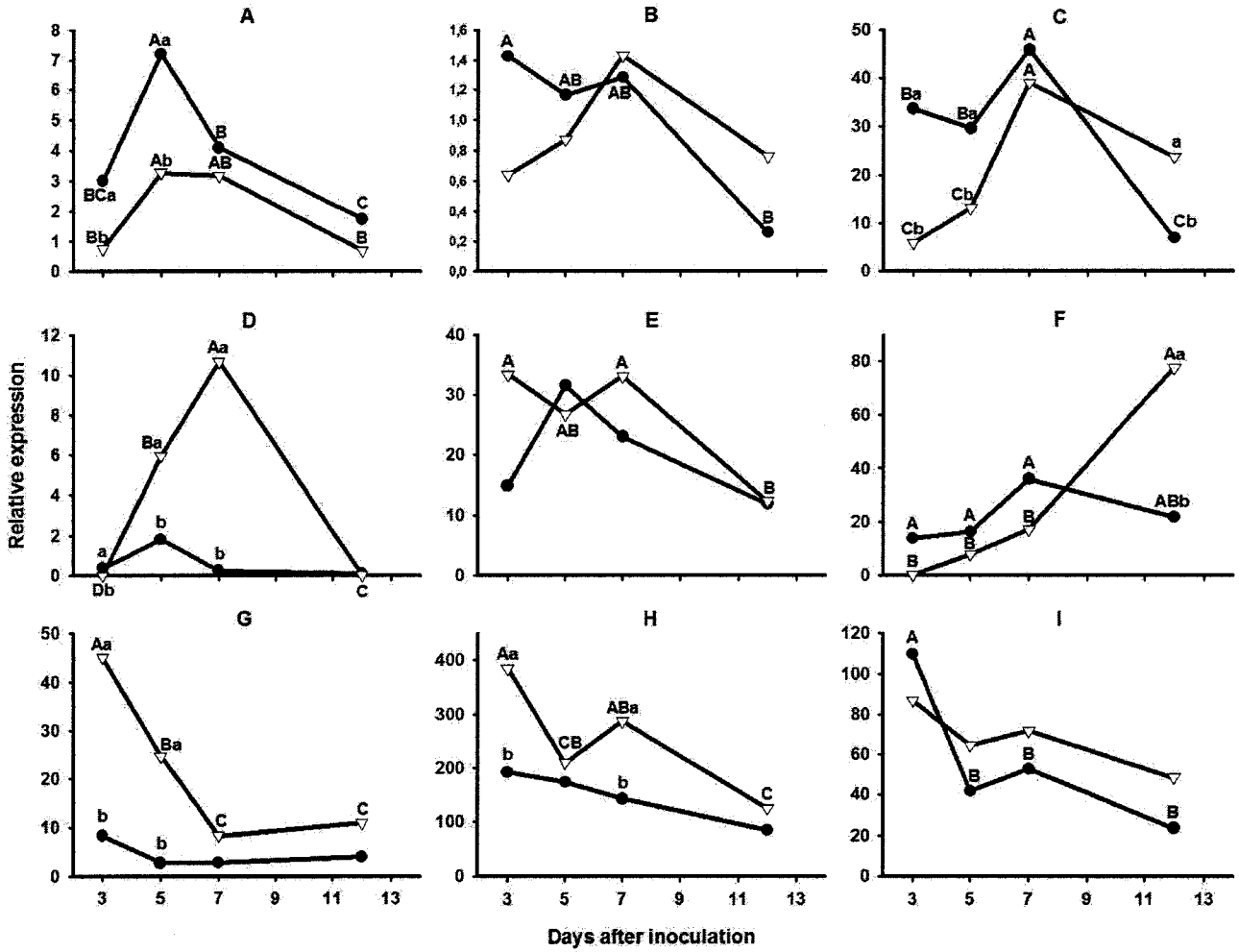

A
B
C
D
E
F
G
H
I
Relative expression
Days after inoculation
3
5
7
9
11
13

Figure 6.2. Gene relative expression of different genes during the interaction of *S. elegans* with live and killed host (*R. solani*) sclerotia at different time periods. A, *acetylcholinesterase*; B, *ankyrin repeat protein*; C, *calmodulin*; D, *cytochrome P450*; E, *mog1*; F, *sechi44*; G, *seglu*; H, *selec*; I, *se151.* The relative expressions were calculated from three replicates using the equation from Pfaffl (2001). Capital and lower case letters on the curves represent significance $P \leq 0.05$, according to LSD, across time and between treatments, respectively. ●, killed host sclerotia inoculated with *S. elegans* conidia; ▽, live host sclerotia inoculated with *S. elegans* conidia.


A
B
C
D
E
F
G
H
I
Relative expression
Days after inoculation
3
5
7
9
11
13

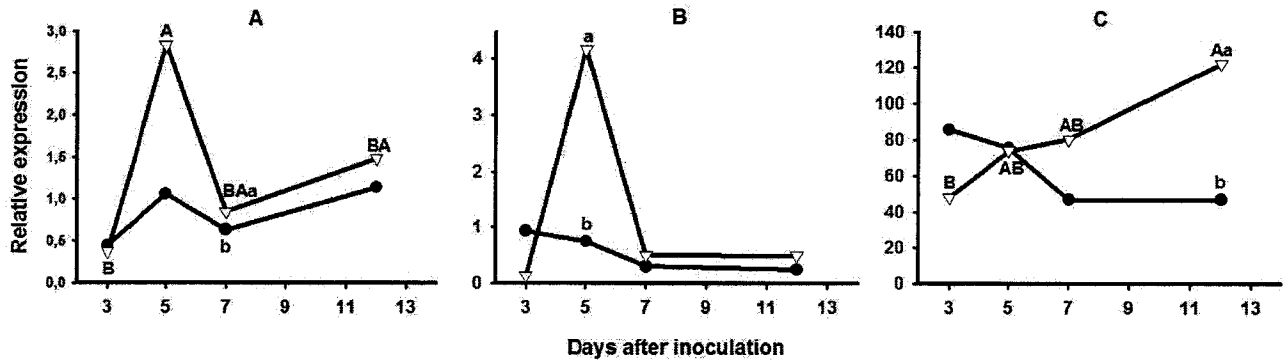


Figure 6.3. Gene relative expression of different genes during the interaction of *S. elegans* with live and killed non-host (*Sc. sclerotiorum*) sclerotia at different time periods. A, *acetylcholinesterase*; B, *ankyrin repeat protein*; C, *se151*. The relative expressions were calculated from three replicates using the equation from Pfaffl (2001). Capital and lower case letters on the curves represent significance $P \leq 0.05$, according to LSD, across time and between treatments, respectively. ●, killed non-host sclerotia inoculated with *S. elegans* conidia; ▽, live non-host sclerotia inoculated with *S. elegans* conidia.

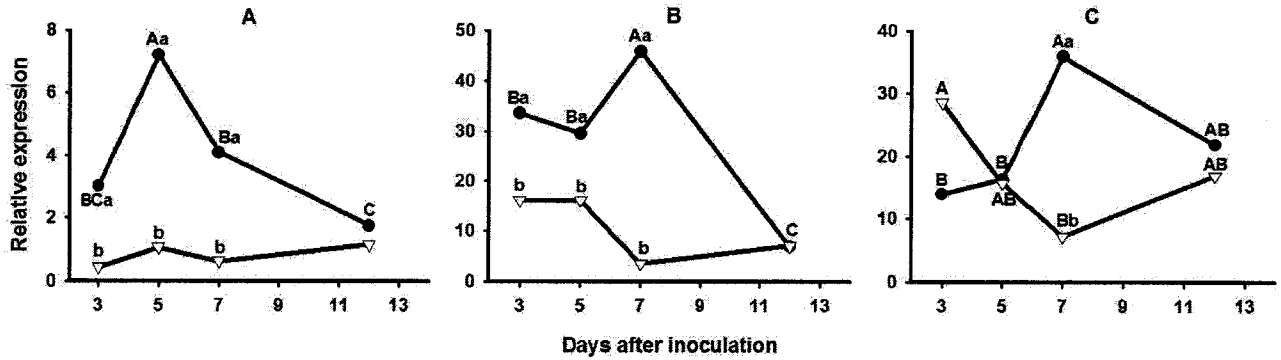


Figure 6.4. Gene relative expression during interaction of different genes during the interaction of *S. elegans* with killed host (*R. solani*) and non-host (*Sc. sclerotiorum*) sclerotia at different time periods. A, *acetylcholinesterase*; B, *calmodulin*; C, *sechi44* The relative expressions were calculated from three replicates using the equation from Pfaffl (2001). Capital and lower case letters on the curves represent significance $P \leq 0.05$, according to LSD, across time and between treatments, respectively. ●, killed host sclerotia inoculated with *S. elegans* conidia; ▽, killed non-host sclerotia inoculated with *S. elegans* conidia.

6.5. DISCUSSION

Expression of selected genes during hyphal interaction between biological control agents and various fungi have been widely described using Northern blot analyses and to a limited extend using QRT-PCR (Cortes et al., 1998; Morissette et al., 2003; Morissette et al., 2006; Rocha-Ramirez et al., 2002; Rotem et al., 1999; Sanz et al., 2005; Zeilinger et al., 1999). However, only few gene expression studies deal with interactions of biological control agents with resting structures such as sclerotia (Giczey et al., 2001). To our knowledge, this is the first report describing the temporal expression of several mycoparasitism-related genes during interaction of a biological control agent with a host and non-host whose sclerotia are anatomically different.

The complete transcript sequences of three novel mycoparasitism-related genes, *seglu, selec,* and *se151* genes, were successfully accomplished through RACE-PCR. Blast analysis of the sequences did not show conclusive functions suggesting that these 3 genes are novel genes. The overtranscription of *seglu*, *selec*, and *se151* appears to be crucial in the early stages of parasitism of either host sclerotia or host hyphae, compared to non-host. However based on the domain sequence analysis, no conclusive role can be assigned for either gene, thus these genes pose new future challenges.

Conidia of several fungal species contain lipophilic self-inhibitors that prevent germination and appressorium formation. Contact with the host's hydrophobic cell surface relieves self-inhibition by diffusion of the self-inhibitors into the plant cuticles (Hedge and Kolattukudy, 1997). Among the genes that are expressed early during germination of C*olletotrichum gloeosporioides* and C. *trifolii* conidia (Buhr and Dickman, 1997; Kim et al., 1998), and appressorium formation of *Magnoprothe grisea* and *C. trifolii* (Liu and Kolattukudy, 1999; Warwar and Dickman, 1996), and whose transcription is affected by self-inhibitors is the calmodulin encoding gene, *cam* (Liu and Kolattukudy, 1999). In our study, *calmodulin* demonstrated different expression patterns based on the type of fungal structure that is parasitized by *S. elegans* conidia. The significant overexpression of *calmodulin* on live host sclerotia compared to non-host sclerotia

suggests that *calmodulin* transcription might be maximally induced by host surface contact and the relieve of self-inhibitors in conidia is due to the surface hydrophobic material known to be present in melanized cells of the sclerotia (Mischke et al., 1995). Of interest, was the substantial *calmodulin* expression on dead host sclerotia compared to live sclerotia. Autoclaving treatment may have caused the hydrophobic material on the surface of the sclerotia to become more accessible, thus relieving self-inhibition and inducing *calmodulin* expression. Relative expression ratios of *calmodulin* during early time-points of interaction with host and non-host hyphae were similar until 96 h after colonization after which *calmodulin* expression was maximally up-regulated during interaction with non-host hyphal cells. This finding is difficult to explain, but it is tempting to hypothesize that *Sc. sclerotiorum* produces inducer molecules that interfere with the metabolism of the mycoparasite.

In the case of *cytochrome P450*, its transcription pattern over time during interaction with non-host hyphae was comparable to that observed when *S. elegans* was alone. However, the substantial increase of its expression in the presence of hyphal and sclerotial cells of *R. solani* strongly suggests that the host produces diffusible molecules that in turn induce *cytochrome P450* expression. In line with this finding, diffusible factors produced by *R. solani* have been reported to substantially trigger the transcription of two chitinases (*chit36* and *ech42*) and a protease (*prb1*) from the mycoparasite *Trichoderma atroviride* (Cortes et al., 1998; Kullnig et al., 2000; Viterbo et al., 2001; Zeilinger et al., 1999).

In yeasts, *mog1* encodes MOG1 protein that is essential for efficient bidirectional nuclear protein import and export and membrane traffic (Baker et al., 2001). Thus, it was not surprising that *mog1* was highly transcribed at early stages during high mycoparasitic activity of host hyphae and sclerotia. On the other hand, its overproduction at later stages of interaction with hyphae and sclerotia of the non-host was not expected. In yeast, the overproduction of the membrane protein Yop-1, whose function in intracellular transport is similar to Mog-1, negatively regulates cell growth leading to accumulation of internal cell membranes and a blockage in membrane traffic (Calero et al., 2001). In our study,

whether the non-host is able to defend itself from attack by eliciting the overproduction of MOG1 to levels that result in accumulation of internal cell membranes and a decrease in nuclear transport in the mycoparasite is worth pursuing.

The expression pattern of the endochitinase encoding gene *sechi44* during interaction with host sclerotia is similar to what we have previously observed during interaction with the hyphae (Morissette et al., 2006) indicating that different types of fungal structures did not affect the expression of *sechi44*, a cell wall-degrading enzyme-encoding gene. On the other hand, this study demonstrated that sclerotia and mycelia trigger different gene expression patterns of some genes. The absence of transcripts of *seglu, selec, se151,* and *ankyrin repeat protein* confirms that these genes are not expressed in the presence of the hyphae of non-host only. This observation strongly suggests that inhibition of gene expression is related to the presence of inhibitor molecules produced by hyphal cells and not by sclerotia of the non-host. How these molecules trigger inhibition of gene expression is not known, and deserves further investigation. Equally, sclerotial development leading to different structural anatomy can affect the mycoparasitic interaction (Willetts and Bullock, 1992), and consequently affect the expression of the mycoparasitic-related genes. In this study, only the expression of some genes such as, *acetylcholinesterase*, *calmodulin*, and *sechi44* were influenced by the structural anatomy of the sclerotia suggesting that a component of the sclerotia induces the expression of these genes.

In conclusion, we have demonstrated that the transcript patterns of several mycoparasitism-related genes of *S. elegans* are differentially elicited not only in response to the presence a host and non-host but in response to the type of the vegetative structure attacked.

ACKNOLWEDGMENTS

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CHAPTER 7

GENERAL CONCLUSION AND FUTURE WORK

Despite intensive research on mycoparasitism in the last two decades, this process is still not well-understood and little is known about the genetic regulation occurring during the mycoparasite/host interaction. In fact, the majority of the reports focus on one aspect of the mycoparasitic process of *Trichoderma* species, the production of CWDEs and the expression of their encoding genes. However, mycoparasites as biocontrol agents are widely accepted but their mycoparasitic efficacy turned out to be variable and not always reliable when tested under field conditions. Thus, a better understanding of the modes of action and genetic regulation of mycoparasites in general might improve their efficacy and strengthen their use as biological control agents. Chapters 3 to 6 of this thesis report gene expression and regulation of the mycoparasite *S. elegans*.

The endochitinase-encoding gene *sechi44* was cloned from *S. elegans* in Chapter 3. Primers were designed based on homology of 42-kDa endochitinase-encoding genes from other mycoparasites and were used in order to amplify a 896-bp segment of the target gene. Specific primers were designed based on this amplicon and the entire open reading frame (ORF) was using RACE-PCR and sequenced. The sequence of the gene *sechi44* showed high homology with the endochitinase-encoding gene *ech42* from the mycoparasite *T. atroviride*. Temporal gene expression was monitored before the contact of the mycoparasite and its host, and after 2, 3, 9, and 12 days of contact. Its expression during interaction showed an increase of transcripts from 2 days through 9 days after contact. However, *sechi44* expression was repressed prior to contact suggesting that *R. solani* produced diffusible molecules that in turn repressed *sechi44* expression. Light microscope observations showed that the germination of *S. elegans* conidia is delayed for 30h on dual culture (*S. elegans*/*R. solani*) compared to a culture of the mycoparasite alone. This result suggests that *R. solani* produced an inhibitor against *S. elegans*. The nature of these diffusible molecules remains

to be determined. This is in line with what other researchers had suggested with the action of the endochitinases *ech42* and *chit36* and protease *prb1* in *T. atroviride* (Cortes et al., 1998; Kullnig et al., 2000; Viterbo et al., 2001).

In order to gain a better understanding of the regulation of *sechi44*, Chapter 4 investigated the influence of various carbon and nitrogen sources incorporated into culture media on the expression of *sechi44* every 24 hours over a period of 12 days. The expression of *sechi44* was substantially increased by the presence of *N*-acetylglucosamine, chitin, and *R. solani* cell wall (simulated mycoparasitism) and is repressed by high concentrations of carbon and ammonium. This latter observation suggested that *sechi44* is under carbon and nitrogen repression and is in line to the findings on the majority of endochitinase encoding genes from *Trichoderma*. Interestingly, the differential expression of *sechi44* followed a two-day cyclical pattern when *S. elegans* was grown in interaction with *R. solani* as well as alone, although the expression was substantially lower in the latter treatment. These findings are novel and have not been observed with *Trichoderma* sp., and strongly suggest the endochitinase-encoding gene is involved in both, growth and mycoparasitism. Whether the expression of *sechi44* is essential for both events remains to be determined by the development and study of knockout strains.

Chapter 5 studied the gene regulation occurring during the *in vivo* mycoparasitic interaction of *S. elegans* with its host *R. solani*. More than one thousand clones were identified as differentially expressed during mycoparasitism by means of suppression subtractive hybridization (SSH) and microarrays, and as much as 120 unique up-regulated genes were isolated. These genes presented a broad range of functions reflecting the complexity of the genetic regulation during this process including the ones related to pathogenesis such as toxin metabolism, pathogenic processes, stress response, multidrug resistance, and apoptosis. Moreover, several novel genes from the mycoparasite were isolated. Among the 43 unidentified genes, three displayed an important induction of their expression during interaction of the two fungi, suggesting that genes other than those encoding CWDEs could be a target to improve mycoparasitic activity and plant

resistance. Furthermore, the results highly suggest that *S. elegans* produces mycotoxins during mycoparasitism, which is a new finding for this species.

For the first time, defense-related genes encoding for transport, vitamin metabolism, and respiration chain processes from the host *R. solani* were isolated. Future experiments targeted at expression and genetic studies of *R. solani* defense genes may provide new insights into the current understanding of what mechanism(s) does the host employ to oppose the attack and defend itself against the spread of the mycoparasite. Since only 26% (261) of the clones have been sequenced, more work is required including the sequencing, characterization and expression analysis of the remaining 755 clones.

The differential and temporal expressions of genes identified in Chapter 5 were monitored in Chapter 6. In addition to the endochitinase-encoding gene *sechi44*, encoding genes whose identification was confirmed as *acetylcholinesterase*, *ankyrin repeat protein*, *calmodulin*, *cytochrome P450*, *mog1*, and the unidentified novel genes *seglu*, *selec*, and *se151*, were studied. The complete ORF of the last three genes were determined using RACE-PCR. Blast on these sequences does not show any conclusive functions suggesting that these three genes are novel genes. For the first time, temporal expression analyses of mycoparasitic-related genes were compared during interaction studies of *S. elegans* with different vegetative structures of a host (*R. solani*) and a non-host (*Sclerotinia sclerotiorum*). Based on light microscope observations, the germination of *S. elegans* conidia appeared 48h followed by the development of appressoria 66 h after spray treatment of the conidia on the *R. solani* cultures. Generally, depending on the target gene under study, regulation occurred at different time periods either very early during the mycoparasitic process or prior to the appearance of infection pegs (*acetylcholinesterase*, *cytochrome P450*, *mog1*) or once the infection pegs appeared (*se151*, *ankyrin repeat protein*, *seglu*, and *selec*) or at later stages in the interaction process of the host (*calmodulin* and *sechi44*). Furthermore, the results demonstrated that *cytochrome P450*, *seglu*, and *selec* play an important role in the mycoparasitic process of the host's hyphae and sclerotia. Interestingly, the expression of four of the target genes (*ankyrin repeat*

protein, *seglu*, *selec*, and *se151*) were substantially repressed in the presence of *Sc. sclerotiorum* hyphae highly suggesting that the non-host produces an repressor(s) targeting these genes. In contrast, the presence of the non-host induced the expression of c*almodulin* and *mog1* on hyphae and ankyrin repeat protein and *sechi44* on sclerotia. Why would the non-host elicit over transcription of these genes is not yet known and remains to be determined.

The characterization or identification of these molecules (inhibitors and inducers) should be investigated by studying the exudates of dual cultures. The results of this chapter also demonstrated that expression of three genes (*acetylcholinesterase*, *calmodulin*, and *sechi44*) is influenced by the ultrastructure of the sclerotia, suggesting that a structural component(s) of the sclerotia stimulates the expression of these genes. The exact role of the above genes, and the inducer and suppressor molecules, are not yet determined. Knockout experiments have to be developed and monitored. These experiments should be set up in such a way that the influence of the knock out of a gene on the other genes is monitored.

CONTRIBUTION TO SCIENCE

The four chapters in this thesis present significant and original contributions to knowledge on the interaction between a mycoparasite and a fungal host mycoparasitic process:

1) Chapter 3 focuses on the cloning and the characterization of the endochitinase-encoding gene *sechi44* from *Stachybotrys elegans*. It is not only the first gene to be cloned from *S. elegans* but the first CWDEs gene in 2003 to be studied and monitored during confrontation of the mycoparasite with its host employing QRT-PCR technique. The characterization of *sechi44* allowed us to draw valid comparisons, with respect to its implication in mycoparasitism, with *ech42*, the most studied endochitinase gene from *Trichoderma atroviride*. The expression of *sechi44* during the interaction of the mycoparasite with its host, *R. solani*, presented similarity with *ech42*, but also difference including a repression in expression prior to contact of interacting hyphae suggesting that the expression of *sechi44* is repressed by *R. solani* before the contact occurred.

2) Chapter 4 reports on the temporal gene expression of *sechi44* under the influence of different carbon and nitrogen sources and during the interaction *S. elegans*/*R. solani*. *sechi44* appeared to be influenced in the same manner as the endochitinase gene *ech42* from *T. atroviride*. However, the study of *sechi44* expression was monitored for the first time over a period of 12 days as opposed to 4 days in previous studies with *Trichoderma* species. The endochitinase encoding gene *sechi44* had a cyclical expression with a peak every 2 days, an expression pattern never reported before in confrontation studies of a mycoparasite with a host. This new evidence strengthened the hypothesis that in addition to its role during mycoparasitism, it is involved in the linear growth of the mycoparasite.

3) Chapter 5 identifies genes that are specifically up-regulated during the interaction between *S. elegans* and *R. solani*. More that one third of these genes are considered novel, while the remaining were not previously reported to be associated with mycoparasitism. To our surprise, none of the isolated genes in the enriched library were identified as CWDE-encoding genes highly suggesting that

these genes are not the most abundantly expressed during mycoparasitism and contrary to what researchers believe. These over-transcribed genes present a potential advancement in understanding the mycoparasitism process and in improving the antagonistic process of mycoparasites. To our knowledge, the results of this study represent the first report on the identification and expression of non-CWDE-encoding genes of a mycoparasite other than *Trichoderma* species as well as the identification of defense genes expressed by *R. solani* in response to attack by a mycoparasite.

4) Chapter 6 describes the temporal and differential gene expression profiles of nine genes, some of which are characterized (*acetylcholinesterase*, *ankyrin repeat protein*, *calmodulin*, *cytochrome P450*, *mog1*, *sechi44*) and some are novel (*seglu*, *selec*, and *se151*). Of interest, the open reading frame of these three novel genes was sequenced. None showed significant homology with known genes and proteins but their expression suggest that they have an important role in the mycoparasitism of *R. solani* by *S. elegans*. Gene expression was monitored during the infection of different types of vegetative structures of a compatible (*R. solani*) and a non-compatible (*Sclerotinia sclerotiorum*) host by *S. elegans*. This study reports on new findings that demonstrate the influence of time, the anatomy of sclerotia, and the nature of the interacting host on gene expression. Interestingly, gene expression is differential according to the vegetative structure of the host (hyphae or sclerotia) parasitized. Thus, some genes appeared to be greatly involved in the mycoparasitism of sclerotia but not in the mycoparasitism of hyphae (e.g., *selec*). Furthermore, complete repression of expression of mycoparasitism-related genes (*seglu*, *selec*, *se151*, and *ankyrin repeat protein*) was shown for the first time to be triggered by a non-compatible host. In addition, the non-compatible host induce the transcription of other genes (*calmodulin* and *mog1*) showing the complexity of gene regulation of the mycoparasitic process.

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APPENDIX

I. SEQUENCE CONTRIBUTION TO GENBANK

AF516397, AY218835, DQ369856, DQ369857, DQ369862, DQ369858, DQ369855, DQ369861, DQ369859, DQ369860, DQ369849, DQ369850, DQ872900, DQ369851, DW714025, DW714026, DQ369798, DW520740, DW520741, DW520742, DW520743, DW520744, DW520745, DW520746, DW520747, DW520748, DW520749, DW520750, DW520751, DW520752, DW520753, DW520754, DW520755, DW520756, DW520757, DW520758, DW520759, DW520760, DW520761, DW520762, DW520763, DW520764, DW520765, DW520766, DW520767, DW520768, DW520769, DW520770, DW520771, DW520772, DW520773, DW520774, DW520775, DW520776, DW520777, DW520778, DW520779, DW520780, DW520781, DW520782, DW520783, DW520784, DW520785, DW714026, DW520786, DW520787, DW520788, DW520789, DW520790, DW520791, DW520792, DW520793, DW520794, DW520795, DW520796, DW520797, DW520798, DW520799, DW520800, DW520687, DW520801, DW520802, DW520803, DW520804, DW520805, DW520806, DW520807, DW520808, DW520809, DW520810, DW520811, DW520812, DW520813, DW520814, DW520815, DW520816, DW520817, DW520818, DW520819, DW520820, DW520821, DW520822, DW520823, DW520824, DW520825, DW520826, DW520827, DW520692, DW520828, DW520829, DW520830, DW520831, DW520832, DW520833, DW520834, DW520835, DW520836, DW520837, DW520838, DW520839, DW520840, DW520841, DW520842, DW520843, DW520844, DW520845, DW520846, DW520847, DW520848, DW520849, DW520850, DW520851, DW520852, DW520853, DW520854, DW520855, DW520856, DW520857, DW520858, DW520693, DW520689, DW520721, DW520736, DW520737, DW520738, DW520688, DW520859, DW520865, DW520866, DW520867, DW520868, DW520869, DW520870, DW520871, DW520872, DW520713, DW520719, DW520720, DW520711, DW520690, DW520712, DW520684, DW520683, DW520694, DW520723, DQ369839, DW520735, DW520861, DW520862, DW520685, DW520863, DW520686, DW520695, DW520696,

DW520697, DW520698, DW520699, DW520700, DW520701, DW520702, DW520703, DW520704, DW520705, DW520706, DW520707, DW520708, DW520709, DW520864, DW520734, DW520739, DW520714, DW520715, DQ369819, DQ369804, DW520716, DW520724, DQ369842, DW520710, DW520873, DW520874, DQ369823, DQ369820, DW520722, DW520717, DQ369826, DQ369806, DW520718, DQ369848, DQ369845, DQ369812, DQ369809, DQ369834, DQ369837, DQ369810, DQ369840, DQ369816, DQ369836, DQ369830, DQ369817, DQ369827, DQ369835, DQ369822, DQ369843, DQ369841, DQ369844, DQ369828, DQ369805, DQ369799, DQ369798, DQ369815, DQ369814, DQ369821, DQ369829, DQ369807, DQ369838, DQ369813, DQ369800, DQ369831, DQ369802, DQ369825, DQ369833, DQ369846, DQ369824, DQ369811, DQ369832, DQ369803, DQ369808, DQ369818, DQ369801, DW520725, DW520726, DW520727, DW520728, DW520729, DW520730, DW520731, DW520732, DW520733, DW520860, DW520875, DW520882, DW520884, DW520888, DW520889, DW520890, DW520892, DW520691, DW520876, DW520877, DW520878, DW520879, DW520881, DW520883, DW520885, DW520886, DW520887, DW520891, DW520893.

II. Light microscope observations of germination of *S. elegans* conidia under different treatments. Conidia were sprayed at a concentration of 105 conidia/ml on a Petri plate containing minimal synthetic media covered with a cellophane membrane. The cultures of *S. elegans* and *R. solani* were 5-day old.

Treatments	Time after spray (hours)							
	9h	18h	24h	36h	48h	60h	66h	72h
Conidia alone	+	+	+	+	+	+	+	+
Conidia on *S. elegans*	n/d	+	+	+	+	+	+	+
Conidia on *R. solani*	-	-	-	-	+	+ CP	+ C, IP	+ C, IP

+, germination; n/d, no data; -, no germination; CP, conidia pressure; C, coiling; IP, infection pegs.